A suite of selective pressures supports the maintenance of alleles of a *Drosophila* immune peptide

Running Title: Antagonistic roles of *Diptericin* alleles

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Abstract

The innate immune system provides hosts with a crucial first line of defense against pathogens. While immune genes are often among the fastest evolving genes in the genome, in *Drosophila*, antimicrobial peptides (AMPs) are notable exceptions. Instead, AMPs may be under balancing selection, such that over evolutionary timescales multiple alleles are maintained in populations. In this study, we focus on the *Drosophila* antimicrobial peptide Diptericin A, which has a segregating amino acid polymorphism associated with differential survival after infection with the Gram-negative bacteria *Providencia rettgeri*. Diptericin A also helps control opportunistic gut infections by common Drosophila gut microbes, especially those of *Lactobacillus plantarum*. In addition to genotypic effects on gut immunity, we also see strong sex-specific effects that are most prominent in flies without functional *diptericin A*. To further characterize differences in microbiomes between different *diptericin* genotypes, we used 16S metagenomics to look at the microbiome composition. We used both lab reared and wild caught flies for our sequencing and looked at overall composition as well as the differential abundance of individual bacterial families. Overall, we find flies that are homozygous serine for *diptericin A* are better equipped to survive a systemic infection from *P. rettgeri*, but in general homozygous arginine flies have a longer lifespan after being fed common gut commensals. Our results suggest a possible mechanism for the maintenance of genetic variation of *diptericin A* through the complex interactions of sex, systemic immunity, and the maintenance of the gut microbiome.

Introduction

An effective immune response is essential for organisms to protect themselves from pathogens. However, an excessive immune response causes damage to self either directly (autoimmunity, cytokine storms) or through dysbiosis *via* disruption of the composition of beneficial microflora [1–4]. The dynamic suite of microbes that hosts face necessitates initiating a robust systemic immune response, avoiding self-harm, and maintaining a beneficial microbiome. The challenge of maintaining a balanced immune response is exacerbated because the threat of microorganisms is contextual; thus, the immune system must distinguish harmful versus beneficial microbes in each context [5,6]. For example, an ingested microbe may be harmless or even beneficial as a food source in the digestive tract, but, when in circulation, that same microbe may prove harmful to the organism [7]. One consequence of balancing a robust innate immune system is that it may lead to the maintenance of genetic polymorphism in genes that encode for proteins intimately involved in the immune response [8,9]. Such patterns of maintained polymorphisms contrast the standard coevolutionary arms race model where hosts and pathogen continually adapt to each other leading to rapid evolutionary change in immune genes [10–12]. Though the coevolutionary arms race model appears apt in many cases, the case for balancing selection on genes involved in the immune defense stems from a more nuanced view of the delicate interplay of systemic immunity, life history traits and the beneficial microbiome [13,14].

Balancing selection is the general term for the adaptive maintenance of multiple alleles in a population. In contrast to the evolutionary arms race model, natural variation in many immune genes is maintained by balancing selection [15–18]. The inference of balancing selection from molecular population genetic data is difficult, especially when genes are small and in areas of

high recombination. Nonetheless, several examples of balancing selection exist, many of which involve small effector proteins of the innate immune system [18–21].

Balancing selection on immune genes is likely to involve allelic benefits that are conditionally beneficial. One way that an allele could be conditionally beneficial is if there is specificity between pathogen and allele [22] such that *allele A* better protects against *pathogen 1* and *allele B* better protects against *pathogen 2*: pathogen specificity. Another way that an allele can be conditionally beneficial in the context of immune defense is if resistance alleles are costly in the absence of infection. In this case, *allele A* better protects against both *pathogen 1* and *pathogen 2*, but in the absence of infection *allele A* is costly for its host [23]. This cost could be energetic or through autoimmune-like damage. In reality, there is likely a continuum between these alternative hypotheses.

Invertebrates lack a traditional adaptive immune system, so the delicate balance between systemic and gut immunity is achieved only through adjustments to the innate immune response [24]. It is therefore important to understand how invertebrates optimize their systemic immune response with as little detriment to their beneficial gut microbiota as possible.

Though first characterized for its role in systemic immunity, the (IMD) pathway is also the main NF-κB immune pathway in the gut and contributes to the maintenance of the microbiome and protection from gut infections [25–27]. To date, there is little research into how individual AMPs help maintain a healthy microbiome composition in the fly, let alone how allelic variation may affect microbiome composition [28,29]. The standard gut microbiome of lab reared *D.*

82 *melanogaster* consists of two prominent bacterial genera: *Lactobacillus* and *Acetobacter* [27].
83 Both genera are easily cultured in the lab, and the *Drosophila* gut can easily be manipulated,
84 making it a good model to study the effects of immune gene variation on microbiome
85 composition and how microbiome composition can, in turn, influence host fitness.
86
87 Antimicrobial peptides (AMPs) are a critical part of the innate immune system that act as broad-
88 spectrum antimicrobials, combating bacteria, fungi, and viruses. In the arms race model, AMPs
89 are on the front lines: directly interacting with microbes. AMPs have also been demonstrated to
90 have critical roles in other physiological functions, for example, dysregulation of AMPs has been
91 connected to diseases such as atopic dermatitis and Alzheimer's disease [2,30,31]. AMPs also
92 contribute to the aging process where the immune system becomes more active as organisms
93 ages to compensate for a decline in its effectiveness. However, this leads to cytotoxicity that in
94 turn shortens lifespan [3].
95
96 In *Drosophila,* AMPs play a crucial in both manage systemic infections and in the maintenance
97 of the gut microbiota [32]. AMPs, however, appear to evolve more slowly than most immune
98 gene families [33–36]. One explanation for this perceived lack of adaptive evolution is that
99 *Drosophila* AMPs genetic variation is maintained adaptively through balancing selection
100 [15,37,38]. Naturally occurring allelic variation in AMP loci are sometimes associated with
101 variability in pathogen resistance [19,39]. *Diptericin A* (hereon referred to as *diptericin* or *Dpt)* is
102 one of the canonical effector genes of the *Drosophila* Immune deficiency (IMD) pathway, and is
103 generally associated with defense against Gram-negative bacterial infection [40,41].
104

105 In both *D. melanogaster* and *D. simulans*, an amino acid polymorphism at the 69th
106 residue of the mature 83-residue Diptericin peptide segregates in most populations surveyed. In
107 both species, this polymorphism is due to a point mutation that changes the ancestral serine (S)
108 allele to arginine (R), but the two species use different codons for arginine. Unckless *et al.*
109 (2016) found a significant difference in survival based on *diptericin* genotype after systemic
110 infection with the Gram-negative bacteria, *Providencia rettgeri,* a natural pathogen of *D.*
111 *melanogaster.* The study used inbred fly lines and found homozygous serine flies have a better
112 survival rate 5 days post infection (~60%) than homozygous arginine flies (~20%) or flies with a
113 premature stop codon in *diptericin* (0%). Later work by Hanson *et al.* took a more general
114 approach to AMP specificity and found that *Diptericin* plays a disproportionate role in response
115 to infection with *P. rettgeri [42].* What remains unclear, however is why this presumably
116 deleterious arginine allele persist in populations of two different species. We hypothesize that it
117 either protects against a different suite of pathogens or it is beneficial in the absence of infection
118 through some life history tradeoff.
119
120 This study aimed to test two hypotheses about the maintenance of genetic variation in *Diptericin.*
121 First, different *Diptericin* alleles might protect against different pathogens – we refer to this as
122 the pathogen specificity hypothesis and it would be supported if the arginine allele were to be
123 associated with higher survival than the serine allele in some infections. In contrast, flies with the
124 serine allele might be generally better at surviving infection, but this may have a cost to the
125 organism. We test whether there are tradeoffs between systemic immunity and other life history
126 traits – particularly traits related to the gut microbiome utilizing genetically controlled lines for
127 *Diptericin* alleles generated by CRISPR/Cas9-editing. Flies were systemically infected with a

128 panel of six different bacteria and homozygous serine flies had a better 5-day survival with each
129 bacterium. We then used axenic and gnotobiotic flies to look at the lifespan of flies since AMP
130 overexpression and microbial proliferation are commonly observed in aging flies [43–46].
131 *Lactobacillus plantarum* is harmful to female flies with non-functional diptericin, while
132 homozygous arginine flies poly-associated with *L. plantarum* and *A. tropicalis* had a longer
133 lifespan than homozygous serine flies. In this way we found evidence for a tradeoff between the
134 ability to fight systemic infection and the ability to control opportunistic gut infections.
135

136 **Results**

137 **A single amino acid change drastically influences survival after infection.**
138 Unckless et al. (2016) found that in inbred lines from the *Drosophila Genetic Reference Panel*
139 (DGRP), flies homozygous for serine as position 69 of the mature Diptericin peptide survive
140 systemic infection with *P. rettgeri* much better than those homozygous for the arginine peptide at
141 the same position. To control genetic background, we used CRISPR/Cas9 genome editing to
142 create both an arginine allele (single nucleotide change, dpt^{S69R}) and multiple null alleles (1 or 3
143 base pair deletions, *Δdpt* flies), as well as control dpt^{S69} (serine at position 69 of the mature
144 peptide) in *diptericin* (Fig S1A, Table S1). The phenotype for our CRISPR/Cas9 edited flies
145 showed striking similarity to the inbred lines. In systemic infection challenges with *P. rettgeri,*
146 dpt^{S69} flies are better protected from infection than dpt^{S69R} flies (p= 5.42e-08) and *Δdpt* flies
147 (p=6.46e-09, Fig 1A). Remarkably, for inbred lines and CRISPR/Cas9 edited lines, survival for

148 five days post infection with *P. rettgeri* for the serine allele lines is 50-60%, with the arginine

149 allele is 10-20%, and less than 5% for null alleles.

150

151 We next challenged the CRISPR/Cas9 edited flies with systemic infection using multiple other

152 Gram-positive and Gram-negative

153 bacteria to determine whether the

154 arginine allele (dpt^{S69R}) protects

155 against some infections better than

156 the serine allele. Such a finding

157 would support the hypothesis that

158 allelic variation is maintained by

159 different alleles providing specific

160 protection against different

161 microbes. However, the differential

162 response to systemic infection with

163 *P. rettgeri* at an OD_{600}=0.1, as

164 described above, remains the

165 largest difference in immune

166 response between *Dpt* genotypes.

167 Dpt^{S69} flies survived better than

168 dpt^{S69R} flies for all systemic

169 infections tested (Fig 1, Table S2).

170 In the Gram-positive bacterial

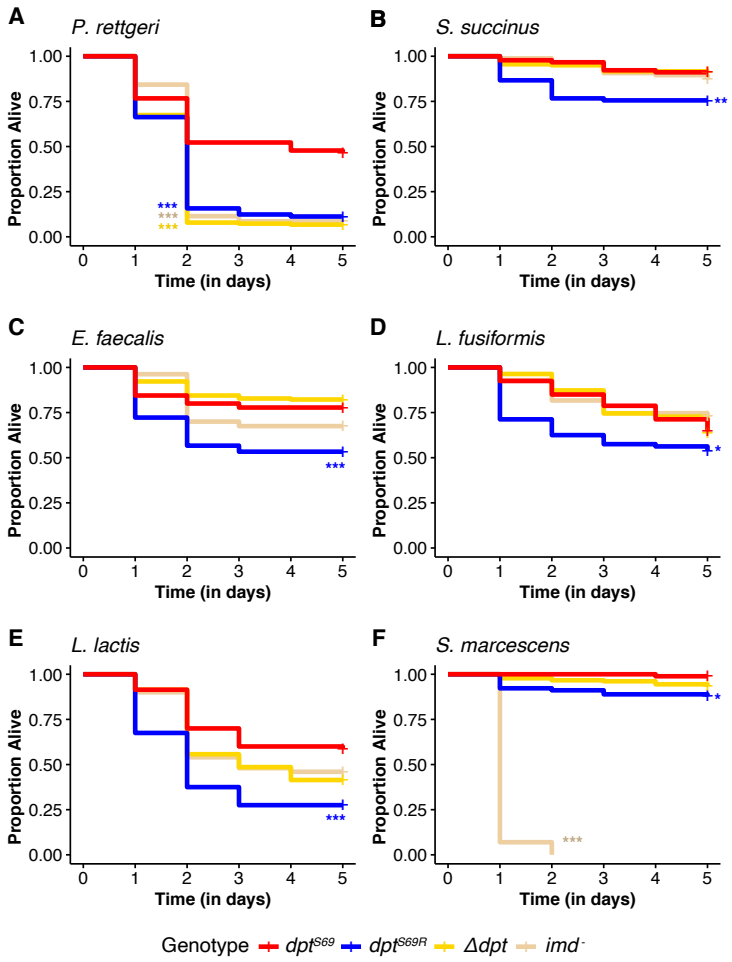

Figure 1: Five-day survival post systemic infection. Survival after systemic immunity challenge with the following bacteria A) *P. rettgeri,* B) *Staphylococcus succinus*, C) *Enterococcus faecalis*, D) *Lysinibacillus fusiformis* Strain Juneja, E) *Lactococcus lactis* F) *Serratia marcescens*. Each graph represents the combined results of 3 different infection dates of at least 20 males of each genotype for each date (at least 60 total). Significance is relative to dpt^{S69} (red line) using Cox proportional-hazards regression model.

171 infections (*Enterococcus faecalis*, *S. succinus, L. fusiformis,* and *L. lactis)*, dpt^{S69R} flies had lower
172 survival than that of *Δdpt* or imd^{-} (null allele for the Imd gene) flies (Fig 1B-E). The only
173 systemic infection where *dpt* genotype did not seem to matter was *S. marcescens,* where all flies
174 survive infection well except for imd^{-}, which die very quickly (Fig 1F).
175
176 We also tested if males and females showed differences in systemic immunity based on genotype
177 for *P. rettgeri, E. faecalis* and *L. plantarum* infections (Fig S2, Table S3). Generally, the *Dpt*
178 genotypes have similar survival patterns between males and females when infected with *P.*
179 *rettgeri.* However, females do have lower 7-day survival rate compared to males (p=0.0039).
180 Females also showed lower survival compared to males after systemic infection with *E. faecalis*
181 (p=0.0003). Higher male survival post infection was observed previously for both *E. faecalis* and
182 *P. rettgeri* with differences ascribed to the Toll pathway [47].
183
184 Overall, flies with the dpt^{S69} allele are better equipped to survive a systemic bacterial infection
185 than dpt^{S69R} flies, though this is most pronounced for *P. rettgeri*. Of the 6 bacteria tested, there
186 was no case where dpt^{S69R} flies survived the infection better than dpt^{S69} flies. These results do not
187 support the hypothesis that alleles are maintained to better combat different pathogens.
188
189 **Diptericin genotype affects lifespan of mono- and poly- associated gnotobiotically reared**
190 **flies.**
191 Although our survey of systemic infections was not exhaustive, we did not find any instances
192 where dpt^{S69R} flies were better able to fight infection, so we turned our attention to the role of
193 *Dpt* in gut microbiome maintenance and immunity. The gut microbiome influences several life

194 history traits in *Drosophila* and other
195 organisms [48–51]. To dissect how
196 *diptericin* genotype influences
197 microbiome maintenance, we
198 manipulated the microbiota in
199 CRISPR/Cas9 flies, and measured
200 longevity and bacterial load. We began
201 with the longevity of axenically reared
202 flies, since it represents a baseline
203 survival without the presence of
204 microbes. Flies with functional copies
205 of *diptericin* (dpt^{S69} or dpt^{S69R}) did not
206 differ in lifespan for either sex
207 ($p_{genotype}$=0.3762), and *Δdpt* lines show a
208 similar lifespan. However, there was a
209 difference in overall lifespan between
210 the sexes among the CRISPR genome
211 edited lines (p_{sex}=1.04e-6). In dpt^{S69} or
212 dpt^{S69R} female flies have a longer
213 lifespan than male flies, as observed
214 generally for *D. melanogaster*
215 previously [52]. In contrast, imd^{-} male

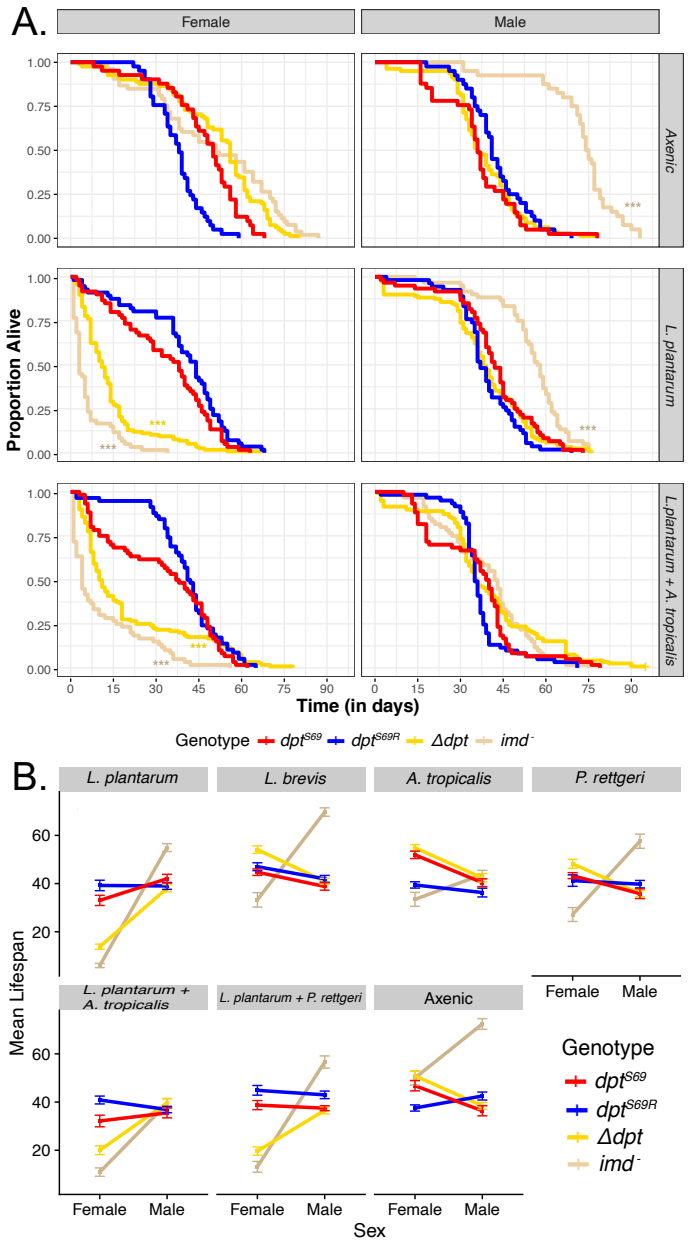

Figure 2: Axenic and gnotobiotic lifespan.
A) Survival plots for axenic flies and gnotobiotic flies mono-associated with L. plantarum or poly-associated with L. plantarum and A. tropicalis. Each curve represents 60 flies from 3 replicates of 20 flies. Significance is shown in comparison to SS (red line) using the model Lifespan~(Genotype*Sex)/Vial+Block. B) Mean lifespan was plotted against each sex and separated by bacterial association. Axenic flies have no bacterial association. Each point for each sex in a combination of 3 separate trials of 20 flies for a total of 60 flies. These interaction plots only have mean lifespan and thus are useful for seeing all the data at a glance.

flies have a much longer lifespan than any of the other lines tested (Fig 2A – axenic row, Table S3).

We next tested the influence of Diptericin genotype on mono-associations with standard constituents of the *Drosophila* gut microbiome. We found multiple sex effects when axenically reared flies were fed *L. plantarum, L. brevis,* or *A. tropicalis* (Figs 2A and 2B, S3). Flies that were fed *L. plantarum* show the most striking differences. Male flies continued to show similar lifespans to each other, but female *Δdpt* and female imd^{-} flies both succumbed quickly post-feeding, indicating functional *Diptericin* is important for gut immunity against opportunistic *L. plantarum* in females.

We also fed axenic flies *P. rettgeri,* which does not readily cause gut infection, to axenically reared flies. Recall that after systemic infection dpt^{S69} flies are more resistant to *P. rettgeri* than dpt^{S69R} flies (Fig 1A). After monoassociation, however, we see no significant difference between the two genotypes with *P. rettgeri* in either sex (p=0.612, Fig 2B). There is also no significant difference between the *Δdpt* and lines with functional Diptericin (Fig 2B, S3). Surprisingly, however, the null allele for *Imd* again shows a significant effect on survival in a sex-specific manner: null *Imd* females die much earlier and null *Imd* males survive much longer when fed *P. rettgeri.*

Some of the sex-specific differences in survival after monoassociation with bacteria may be driven by intrinsic differences in feeding rates between the sexes (and potentially genotypes as well) [53]. To determine whether the differences in male and female survival and load were due

239 in part to differences in feeding rates, we performed a feeding rate assay with blue dye mixed
240 with media (Luria-Bertani broth) or *P. rettgeri* (OD_{600}=15.0). We noted that females did eat
241 more in a single hour of feeding, but that there was also an effect of *diptericin* genotype (with
242 null flies eating less, Figure S4). Thus, it is possible that differences in longevity after
243 monoassociation are due to different rates of exposure to those bacteria because of different
244 feeding rates.
245
246 We next looked at how poly-associations with bacteria affected lifespan. First, we fed flies a 1:1
247 mixture of *L. plantarum* and *A. tropicalis*, two common gut microbes found in lab-reared and
248 wild caught flies [54,55]. We found that female *Δdpt* and imd^- flies had a much shorter lifespan
249 than flies with functional Diptericin (Fig 2A and 2B). This is the same pattern observed in mono-
250 association with *L. plantarum*. However, we observe that *diptericin* genotype influences survival
251 when poly-associated with *L. plantarum* and *A. tropicalis*. Dpt^{S69R} female flies live longer than
252 dpt^{S69} female flies (Fig 2A and 2B; p=0.00782). This is the opposite of systemic infections,
253 where dpt^{S69} flies always survived better than dpt^{S69R} flies, and may indicate a tradeoff between
254 defense against systemic and gut immunity.
255
256 In poly-association with 1:1 *L. plantarum* and *P. rettgeri,* we observe many of the same patterns
257 as in the poly-association with *L. plantarum* and *A. tropicalis*. Again, female *Δdpt* and imd^- flies
258 have a shorter lifespan than female dpt^{S69} and dpt^{S69R} flies (Fig 2B), and *dpt* genotype is
259 associated with lifespan. Dpt^{S69R} flies have a longer lifespan than dpt^{S69} flies (p=0.000198, Fig
260 2B) in both sexes.
261

262 Given the genotypic and sex effects on survival after oral association with *L.* plantarum, we also
263 looked at sex differences after systemically infecting conventionally reared flies with *L.*
264 *plantarum*. We saw no difference in survival between the sexes, as observed for systemic
265 infections with *E. faecalis* or *P. rettgeri* and saw no difference between *Δdpt* lines and lines with
266 functional Diptericin, as observed for axenic flies mono-associated with *L. plantarum* (Fig S2).
267 This could indicate that Diptericin plays different roles for systemic and gut immunity in relation
268 to *L. plantarum* in each sex. Further, dpt^{S69} male flies survived better than dpt^{S69R} male flies
269 (p=0.00438), in line with observations from other systemic infections in males (Fig 1).
270
271 Overall, we found a role of both specific *diptericin* genotype (serine vs. arginine) and the
272 presence of functional copies of *diptericin* for survival after introducing common gut microbes in
273 controlled conditions. Most striking was the sexually dimorphic role of both *Dpt* and *Imd-,* with
274 females being much more sensitive to genotype than males.
275
276 **Gnotobiotic fly bacterial load**
277 Given the differences in survival among sexes and genotype for different gnotobiotic
278 associations with bacteria, we determined whether bacterial load after associations also were
279 different among sexes and genotypes. To assess the influence of genotype on the immune
280 response of aging flies, we studied how well common gut bacteria colonized the gut over 20
281 days, representing ~20-33% of *D. melanogaster's* normal lab lifespan [56,57]. We generated
282 gnotobiotic flies by feeding specific bacteria for two days and observed the bacterial load 2-, 10-
283 and 20-days post feeding.
284

When specifically looking at mono-association with *L. plantarum* we observe that bacterial load differences between genotypes occur within the first 2 days post feeding but begin to disappear by day 20 ($p_{genotype(day2)}$=2.6e-5, $p_{genotype(day20)}$=.709, Fig 3A, S5). Note that in the first two days, the flies were raised on microbe-contaminated media, but after 2 days were moved onto sterile food and then transferred to new sterile food every 3 days. This corroborates what we saw in the longevity data in females. Within the first 15 days a large proportion of *Δdpt* female flies died, and we observed a higher bacterial load in these flies, especially on day 2 post feeding. By day 20, differences in bacterial load in females disappeared. Note that there is inherent sampling bias, as only the flies able to survive until day 20 are sampled at that time point. In the case of *imd*$^{-}$ flies, no females survived until day 20, hence there is no data for *imd*$^{-}$ flies on day 20 (Fig 3A, S5).

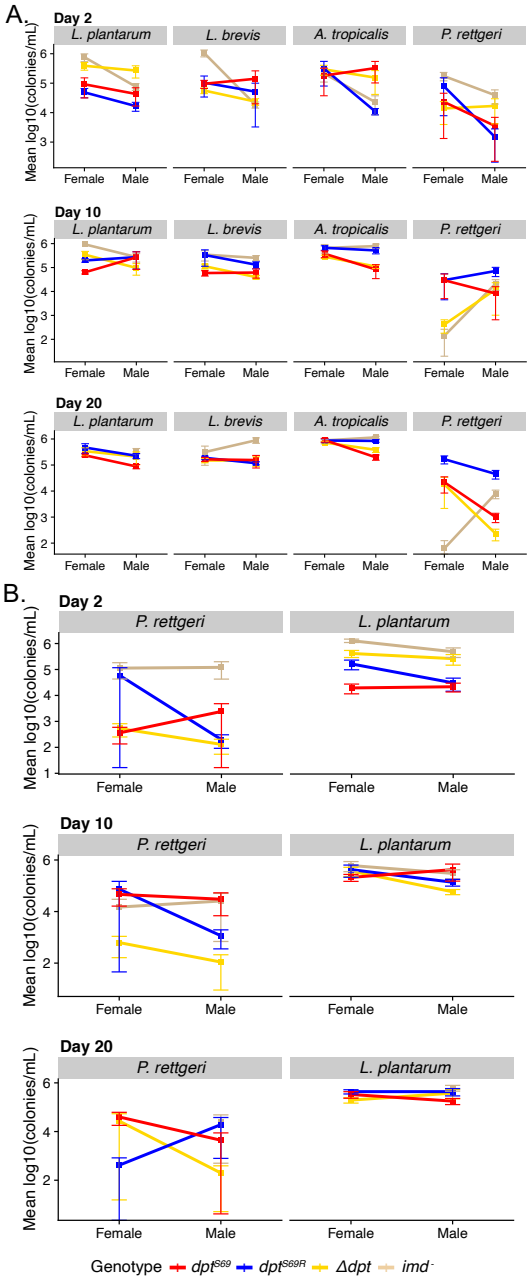

Figure 3: Bacterial load interaction plots for day 2, 10, and 20 post association with various microbes. Two-day old flies were placed on food seeded with a bacterial suspension of each bacteria at an OD600 of 15. Each point for each sex is from 3 separate trials of 2 samples of 3 flies each for a total of 6 samples per point. A) monoassociations where each plot represents a different experiment, B) polyassociations where each row plots both the *P. rettgeri* and *L. plantarum* load from the same flies but were plated on LB and MRS respectively.

307 *P. rettgeri* is the only canonical systemic pathogen we included in our gnotobiotic study, and the
308 patterns based on genotype were different between systemic infection and the gnotobiotic oral
309 infection data. When flies were fed *P. rettgeri,* we saw a range of bacterial loads, from zero
310 colonies to bacterial load levels on par with common gut bacteria. We also observed a genotype
311 effect between dpt^{S69} and dpt^{S69R} on day 10- and 20- post feeding in males ($p_{genotype(day10)}$=0.0044,
312 $p_{genotype(day20)}$=0.0004, model only included dpt^{S69} and dpt^{S69R}, Table S5). In both instances,
313 bacterial load in dpt^{S69R} flies is higher than in dpt^{S69} flies. This may indicate that dpt^{S69} flies are
314 better equipped to deal with both systemic and oral infection from *P. rettgeri*. Whether this is
315 due to a greater ability of dpt^{S69} flies to withstand the effects of infection (tolerance) by *P.*
316 *rettgeri* by remains a question. It is also important to note that *P. rettgeri* establishes poorly in
317 the gut of wildtype flies, which may explain the noisy results for mono associations after oral
318 infection.
319
320 A range of bacterial loads were also observed when flies were poly-associated with *L. plantarum*
321 and *P. rettgeri* (Fig 3B, S6). There were no statistically significant differences between dpt^{S69}
322 and dpt^{S69R} bacterial for this poly association (Table S6). In fact, there were no significant
323 differences between any of the *P. rettgeri* bacterial loads, however *L. plantarum* does show
324 differences on day 2-post feeding (p=7.97e-6). These differences are mainly between flies with
325 non-functional *diptericin* (*imd-* and *Δdpt*) and flies with functional *diptericin*. Both *Δdpt* and
326 *imd-* flies had higher bacterial load than dpt^{S69} and dpt^{S69R} flies on day 2 post feeding which may
327 be an indication of the reason both these lines quickly succumb to feeding with *L. plantarum* at
328 least in the context of the poly-association with *P. rettgeri*.
329

330 We observed larger differences in bacterial load shortly after feeding with bacteria and those
331 differences became less by day 20-post feeding. We did not see any differences in *P. rettgeri*
332 load when mono-associated or when part of a poly-association, indicating the flies respond
333 differently to the same pathogen when introduced systemically or orally.
334

335 **Evidence for life history tradeoffs mediated by *diptericin* genotype**

336 Proteins involved in a robust
337 immune response may have
338 pleiotropic effects on other
339 traits either because of a
340 direct interaction with that
341 trait or because of inherent
342 costs of immune defense
343 (self-damage, energy
344 expenditure, etc.) [58–60].
345 We examined three life
346 history traits (desiccation
347 stress survival, starvation
348 stress survival, and
349 uninfected longevity) to
350 determine whether *Dpt*
351 genotype had such
352 pleiotropic effects.

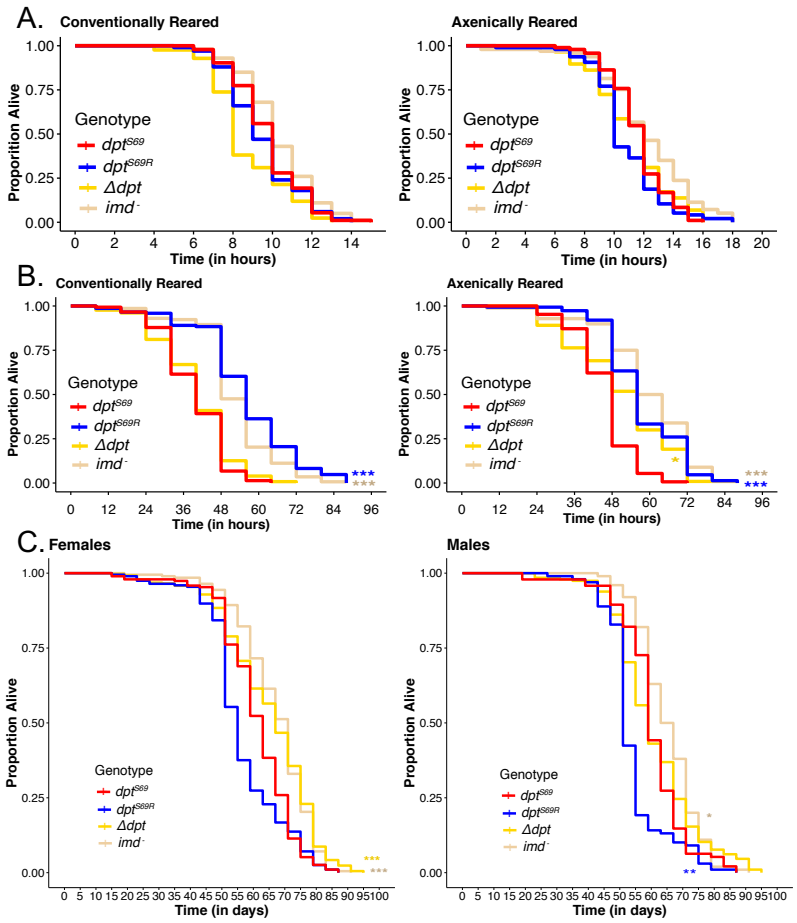

Figure 4: *Dpt* genotype is associated with variation in some life history traits
A) Conventionally and axenically reared male flies desiccation resistance (N>90 for each line apart from *Δdpt* line). There is no significance between dpt^{S69} (red line) and other genotypes. B) Conventionally and axenically reared male flies starvation resistance (N>90 for each line apart from *Δdpt* line). C) Female and male lifespan for conventionally reared flies. N>93 for each line and sex. Significance is shown in relation to dpt^{S69} (red line). Note that axenic longevity is shown in Figure 2. *P<0.05, **P<0.01, ***P<0.001.

353

354 The ability to survive desiccation stress is an important life history trait for wild *Drosophila*
355 survival [61,62]. When subjecting our male CRISPR/Cas9 flies to desiccation, we observe
356 conventionally reared flies succumb faster to desiccation stress than axenically reared flies (Fig
357 4A, $p_{genotype} < 2e\text{-}16$). In conventionally reared males, dpt^{S69} flies have similar desiccation
358 resistance to dpt^{S69R} flies (p=0.917). However, dpt^{S69} flies survive desiccation stress better than
359 dpt^{S69R} flies when reared axenically ($p_{genotype}=0.04126$). We also compared *Drosophila* OreR and
360 W1118 (both homozygous for the serine allele of Diptericin) to the dpt^{S69} line (Fig S7, Table S7).
361 Unsurprisingly, despite the same *diptericin* genotype, all 3 lines show dramatically different
362 desiccation resistance. Therefore, *diptericin* genotype plays limited, if any role in variation in
363 desiccation resistance, as all 3 wildtype lines were derived from different genetic backgrounds.
364

365 We next looked at the effect of genetic variation in *diptericin* on male ability to survive under
366 starvation stress. As with desiccation resistance, we found axenically reared flies survive
367 starvation stress better than conventionally reared flies (Fig 4B, S8, $p_{treatment} < 2e\text{-}16$). However,
368 unlike the desiccation stress conditions, both conventionally reared and axenically reared dpt^{S69R}
369 flies survive starvation stress longer than dpt^{S69} flies (conventional: $p_{genotype} < 2e\text{-}16$, axenic:
370 $p_{genotype} < 2e\text{-}16$). In conventionally reared flies there is no difference in survival between dpt^{S69}
371 flies and *Δdpt* flies (Fig 4B, Table S8). However, in axenically reared flies, *Δdpt* flies have an
372 intermediate survival phenotype between dpt^{S69} and dpt^{S69R} flies. This may suggest an interaction
373 between functional *diptericin* and the microbiome that influences starvation.
374

375 Finally, we looked at overall longevity of female and male conventionally reared flies (in the
376 absence of any infection or other significant selection pressure). Female flies have a longer
377 lifespan than males (Fig 4C, S9, p_{sex}= 5.80e-13) regardless of *dpt* genotype. In males, dpt^{S69} flies
378 had a significantly longer lifespan than male dpt^{S69R} flies (mean of 60.3 days for dpt^{S69} and 54.1
379 for dpt^{S69R}, p= 0.0072), but not in female flies (mean of 61.9 days for dpt^{S69} and 59.0 for dpt^{S69R},
380 p= 0.6434). However, in axenically reared flies only female dpt^{S69} flies have a longer lifespan
381 than male dpt^{S69R} flies. Interestingly, the female flies with the longest lifespan have non-
382 functional Diptericin (Fig 4C, *Δdpt* line and imd^{-} line, Table S9). Males lacking functional
383 Diptericin show the same effect, but to a lesser extent. These results provide evidence that, in the
384 presence of a standard gut microbiota, both *Dpt* and a functional Imd pathway may decrease
385 longevity. This is consistent with others who found downregulation of NF-κB pathways and
386 AMPs increased lifespan in *Drosophila* [60,63], but the fact that flies with *Dpt* null alleles alone
387 are sufficient to increase lifespan is noteworthy.
388

389 **Diptericin's influence on gut microbial diversity**

390 To determine whether *Diptericin* genotype influences the composition of the bacterial
391 community in the gut, we sequenced amplicons of 16s ribosomal rRNA in conventionally reared
392 lab flies under two different rearing conditions: flies reared in standard *Drosophila* vials, and the
393 progeny of the cross between dpt^{S69} and dpt^{S69R} flies reared in cages for more than 2 generations.
394 First, we found flies that were co-reared in cages had similar microbiomes, with no discernable
395 differences by the alpha diversity metric, Shannon diversity (p=0.5239, Fig 5A). On the other
396 hand, the microbiomes of flies reared in vials was distinctly different overall compared to the
397 microbiomes of flies reared in the cages (p<0.001, Fig 5B). However, the differences between

genotypes were still minimal in the vial-reared flies and vial may be a large factor in differences between lines. There were 2 dpt^{S69R} lines used and even though these are the same genotype and genetic background there was a significant difference between the 2 lines (Fig 5B).

The lab is a controlled environment that allows for excellent control of variables but does not replicate the conditions found in nature. Thus, we looked at the microbiomes of wild caught flies collected from the decaying fruit of an apple orchard. We found flies with homozygous for dpt^{S69R}, homozygous for dpt^{S69}, and heterozygous genotypes at our collection location (Table S10). Out of the 955 *D. melanogaster* flies successfully genotyped, only 20 flies were homozygous for dpt^{S69R} were identified. All 20 homozygous dpt^{S69R} flies were profiled along with 36 each of dpt^{S69} homozygous flies and heterozygous flies for a total of 92 flies profiled (amplicon sequences of both *Dpt* and 16s rRNA are in Table S2).

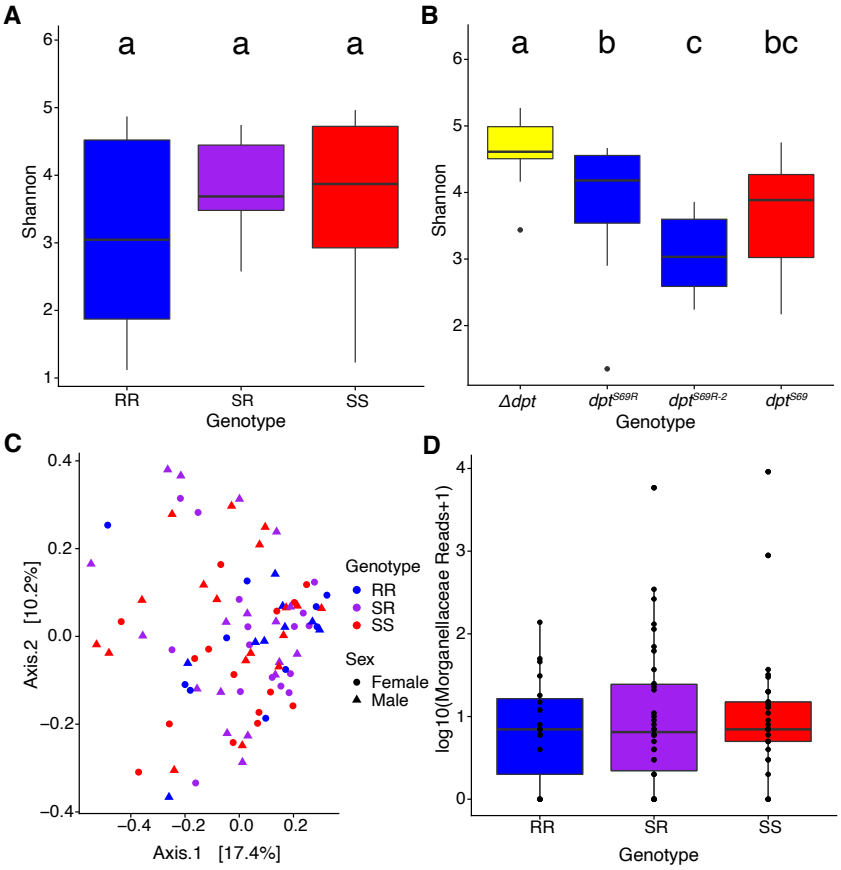

Figure 5 The influence of *Dpt* genotype on the gut microbiota
A) Shannon diversity of flies co-reared in cages. B) Shannon diversity of individual genotypes reared in vials. C) Bray-Curtis dissimilarity of wild caught flies. D) Differential abundance of Morganellaceae in wild caught flies. Different letters show significance based on post-hoc Tukey test, P<0.05.

421

422 We found that *dpt* genotype does not affect the overall composition of the microbiome of wild
423 caught flies based on Shannon diversity (alpha diversity) and Bray-Curtis dissimilarity (beta
424 diversity) (Fig 5C). However, when looking at differential abundance of individual bacterial
425 families there are differences based on genotype. We tested specific association between
426 *diptericin* genotype and normalized counts from 9 microbial families. Most intriguing is the
427 difference in abundance of *Morganellaceae* family reads since, *P. rettgeri* belongs to this family.
428 Heterozygous and homozygous serine flies have a slightly higher abundance than homozygous
429 arginine flies (Fig 5D).

430

431 Overall, our results for associations between *Dpt* genotype and gut microbiome in both lab and
432 wild flies are weak. Such associations may require much larger sample sizes if the effects are
433 small – particularly given the noisy phenotype in the wild. Alternatively, the influence of *Dpt*
434 genotype on gut microbiome diversity may be specific to developmental life stages or microbes
435 that are relatively rare in these populations.

436

437 **Discussion**

438 The adaptive maintenance of multiple alleles of a single gene has been posited for nearly a
439 century [64–68] and evidence for its pervasiveness continues to grow [69–74]. However, there
440 are relatively few cases where we understand the adaptive benefits of both allele (sickle-cell
441 anemia and malaria resistance being the best studied example [75]). In this study, we generated
442 CRISPR/Cas9 genome-edited flies of different *diptericin* genotypes to investigate mechanisms of
443 maintenance of allelic variation in AMPs. This allowed us to specifically study a single amino

444 acid change on an otherwise genetically-controlled background. Overall, we found evidence that
445 *dpt*^{S69} flies survive systemic infection better, while *dpt*^{S69R} flies survive some opportunistic gut
446 infections better. Thus, more robust defense against systemic infection appears to interfere with
447 maintaining a balanced gut microbiota – this tradeoff may result in natural populations
448 maintaining both alleles. Importantly, we note that while our results are consistent with the
449 adaptive maintenance of alleles in natural populations, these lab assays do not prove the adaptive
450 value of different alleles in the field.
451
452 Systemic infections with *P. rettgeri* in CRISPR/Cas9 genome-edited lines confirmed the amino
453 acid polymorphism in Diptericin as the basis for the difference in immune response of flies with
454 different *diptericin* genotypes previously hypothesized *via* an association study [19].
455 Surprisingly, *dpt*^{S69} flies had a higher survival five days post infection than *dpt*^{S69R} flies in *all*
456 systemic infections tested. This may mean either that the serine allele provides improved defense
457 against all the tested systemic infections, or that alterations to other aspects of fly physiology
458 (notably the gut microbiota) could predispose arginine flies to poorer survival. We did not
459 perform systemic infections on axenically-reared flies, but if Dpt genotype influences the
460 microbiome, which in turn influences survival after infection with other pathogens, such an
461 effect should disappear in flies reared without a microbiome.
462
463 Our study also reveals an important role of Diptericin in preventing opportunistic gut infections
464 by common gut microbes. This is especially evident when looking at lifespan after associating
465 axenic flies with *L. plantarum* in both mono- and poly- association contexts. In both sexes,
466 *dpt*^{S69R} flies have a longer lifespan than *dpt*^{S69} flies, and this effect is enhanced in poly-

467 association with *L. plantarum* and *A. tropicalis.* Other studies have posited a role for individual
468 AMPs in the maintenance of gut microbes in *Drosophila* [29], but this appears to be the first
469 example linking a functional copy of a single AMP to survival differences.
470
471 One of our most striking and unexpected results was the distinct sex differences in longevity
472 after association with common gut bacteria. Furthermore, fly genotype plays a significant role in
473 these differences. After association with *L. plantarum*, *imd-* females had the shortest mean
474 survival, while *imd-* males had the longest mean survival. The effect for *Δdpt* was similar:
475 females had much shorter average survival than the other two *dpt* alleles, but the three alleles
476 showed equivalent longevity in males. While the *imd-* sexual dimorphism is relatively consistent
477 across experiments with different microbes, the *Δdpt* effect seems to be more limited to
478 associations involving *L. plantarum.* This result not only highlights the importance of using both
479 sexes in microbiome research, but also confirms that functional Diptericin is important for
480 *Drosophila melanogaster* gut health. There is a growing body of literature on the effects of
481 sexual dimorphisms in immune response, but it is lacking for sexual dimorphisms in gut
482 immunity and our findings only emphasize the need to fill this gap (*Drosophila* immunity sexual
483 dimorphisms reviewed in Belmonte et al. 2020; also see [76]). Our feeding assay provides some
484 evidence that the differences between male and female survival after exposure to microbes is due
485 the amount of food (and therefore the number of microbes) consumed, but these differences
486 should be examined in much more detail. More generally, the sex by genotype effect suggests a
487 likely explanation for the maintenance of genetic variation: different fitness effects of alleles in
488 the different sexes [77]. Note that while our study focuses on the serine/arginine polymorphism,
489 at least 6 null alleles of *dpt* segregate in natural populations of *D. melanogaster* and the

490 frequency of those nulls shows clinal variation in both North American and Africa [78], thus the
491 nulls are likely maintained selectively too.
492
493 Herein we highlight 3 main roles of Diptericin in *Drosophila*. 1) Diptericin genotype influences
494 systemic immune defense; 2) in certain conditions Diptericin genotype influences gut immunity;
495 and 3) Diptericin has sex-specific effects in the gut. These results highlight the need for
496 individuals and populations to modulate the immune system to balance systemic and gut
497 immunity, and how the different needs of females and males complicate this balance. The
498 dramatic differences in survival of males and females in response to oral infection by common
499 gut bacteria underlines the importance of looking at both sexes when examining the maintenance
500 of genetic diversity, as balancing selection may be caused by sexual dimorphism. Overall, our
501 results suggest that a complex interaction between sex, environmental context (starvation,
502 pathogen exposure – both systemic and oral), and genotype may contribute to the long-term
503 maintenance of immune alleles.
504

505 **Materials and Methods**

506 **Drosophila Lines and Rearing**

507 Conventionally reared flies were maintained in a 23°C incubator with a 12h light:12h dark
508 schedule on a cornmeal-molasses-yeast diet (64.3g/L cornmeal, 79.7mL/L molasses, 35.9g/L
509 yeast, 8g/L agar, 15.4mL of food acid mix (50mL Phosphoric Acid + 418mL Propionic Acid +
510 532mL deionized water) and 1g/L Tegosept. We used CRISPR/Cas9 genome editing to modify
511 the *diptericin A* gene. Briefly, DNA coding for guide RNA (gRNA) was inserted into the pUS-
512 BbsI plasmid (Table S1). A single stranded donor DNA (120bp) containing the desired edit (to

513 change from serine to arginine) and a silent mutated PAM site was synthesized by IDT
514 (Coralville, IA, USA). The plasmid and ssODN were then injected into Bloomington stock
515 #55821, which expresses Cas9 driven by the *vasa* promoter, by Genetivision, Inc. (Houston, TX,
516 USA). Individual flies developed from the injected embryos were collected and crossed with a
517 modified version of Bloomington stock # 7198 (a line with *CyO*/*Kruppel* balanced on the 2nd
518 chromosome, and *serrate*/*Dichaete* balanced on the 3rd chromosome). Our version, 7198^{A4}, was
519 provided by Stuart Macdonald and has the DSPR [79] A4 line's X chromosome instead of the
520 *w*[*] from the original 7198. The F1s were collected and again individually crossed with 7198^{A4}
521 for F2 crosses yielding individuals with a homozygous 2nd chromosome representing one of the
522 chromosomes carried by the original injected embryo. The Dpt gene was sequenced from the F2
523 cross progeny to determine whether edits occurred. This yielded several classes of alleles
524 including homozygous serine *dpt* (wildtype, dpt^{S69}), homozygous arginine *dpt* (dpt^{S69R}), and *dpt*
525 null (*Δdpt,* refers to lines with either 1 or 3 base pair deletion) (Fig S1A). Balancers were
526 removed, and lines were moved into the same genetic background through a series of crosses as
527 shown in Fig S1B. An *imd*- line was used as an IMD pathway negative control but note that this
528 line was from a completely different genetic background from the rest of the lines.
529

530 **Axenic Fly Preparation**

531 Microbe-free (axenic) lines were generated by first washing embryos in a 10% bleach solution to
532 dissolve the chorion for 2 minutes. The embryos were then washed in 70% ethanol for 30
533 seconds and water for another 30 seconds, then transferred to autoclaved molasses food (see
534 above). Some embryos from each treatment were placed onto De Man, Rogosa and Sharpe
535 (MRS) agar plates and incubated at 30°C for 48 hours to check that they did not contain viable

536 microbes. Axenic lines were continuously checked for the presence of contaminating microbes
537 (every 3-4 generations) by homogenizing flies and plating the homogenate on MRS agar.
538
539 Axenically and gnotobiotically (see below) reared flies were maintained in an incubator that was
540 isolated from conventionally reared flies. The incubator was kept at 23°C with a 12h light:12h
541 dark schedule. Axenic and gnotobiotic flies were kept on the same molasses diet that had been
542 autoclaved before dispensing into autoclaved vials. Axenic and gnotobiotic flies were only
543 handled inside a sterile hood (Baker SG 400, The Baker Company Inc., Sanford, ME).

544

545 **Bacterial Strains**

546 The following bacteria were used for systemic infection assays: *Providencia rettgeri* [80],
547 *Providencia burhodegraneria* Strain B [80], *Enterococcus faecalis* [80]), *Serratia marcescens*
548 [39], *Lysinibacillus fusiformis* Strain Juneja [81], and *Staphylococcus succinus* (isolated from
549 wild *Drosophila,* Unckless lab). Bacteria were grown from glycerol stocks on LB plates at 37°C
550 overnight.
551
552 The following bacteria were used in gnotobiotic experiments: *L. plantarum, L. brevis, A.*
553 *tropicalis.* All these strains were isolated from plating conventionally reared flies on MRS agar
554 in the Unckless lab at the University of Kansas. Individual colonies from plated fly homogenate
555 were grown overnight in MRS for DNA isolation. Bacterial species were identified using Sanger
556 sequencing with the 16S rRNA region primers 27F (AGAGTTTGATCCTGGCTCAG) and
557 1492R (CGGTTACCTTGTTACGACTT). We also utilized the same *P. rettgeri* as described for
558 systemic infection.

559

560 **Survival Assays**

561 For systemic infections, individual colonies of bacteria were picked and grown in 2mL LB broth
562 shaking overnight at 37°C. Bacterial suspensions were then diluted or concentrated to OD_{600}=0.1
563 for *P. rettgeri,* OD_{600}=10 for *S. succinus,* OD_{600}=1.5 for *E. faecalis*, OD_{600}=3.5 for *L. fusiformis*,
564 OD_{600}=1.0 for *L. lactis*, and OD_{600}=4.0 for *S. marcescens*. *L. plantarum* was grown in 5mL
565 MRS at 30°C overnight and was concentrated to OD_{600}=10 for systemic infections. To induce
566 systemic infection, 5-9 day old, conventionally reared flies were pricked in the thorax with a
567 needle dipped in a bacterial suspension [82]. Infections were done in triplicate with at least 20
568 flies for each replicate per line for a total of 60 flies per genotype per condition. Flies were
569 incubated at 23°C with a 12h light:12h dark schedule and survival was tracked daily for 5 days
570 post infection.

571

572 **Gnotobiotic Longevity**

573 Axenically reared flies were collected within 24 hours of eclosion. Flies were then kept on sterile
574 food for 2 days before sorting for longevity experiments. To begin longevity experiments, flies
575 were separating into groups of 10 individuals of each sex and put onto sterile food seeded with
576 50uL of bacterial suspension at an OD600 of 15 +/- 1. For each replicate, we used 2 vials of 10
577 flies each per sex per line for a total of 20 flies for each sex per genotype for a total of 60 flies
578 across all replicates. Flies were allowed to feed in the inoculated vials for 3 days before being
579 transferred to uninoculated sterile food vials. Flies were flipped to new sterile media every 4-5
580 days for the remainder of the experiment. Surviving flies were counted every 1-3 days until all
581 flies were dead.

582

583 **Gnotobiotic Bacterial Load**

584 To determine whether microbes became established in the gut, we homogenized flies during and
585 after the exposure and plated the homogenate. We measured bacterial load by inoculating flies in
586 the same manner as gnotobiotic longevity. Flies were separated into groups of 5 females and 5
587 males per vial. For 2-day experiments flies were kept on the seeded food for the entire
588 experiment. For 10– and 20- day experiments, flies were allowed to feed on the seeded food for
589 3 days before being transferred to sterile food. Flies continued to be transferred to new sterile
590 media every 3-4 days until day 10 or 20 post feeding. After the experimental (2, 10 or 20 day)
591 time period, flies were surface sterilized by washing in 70% ethanol followed by molecular grade
592 water. Flies were separated by sex and three individuals were homogenized together in 300uL of
593 sterile 1x PBS and the homogenate was plated on the appropriate media using a Whitley WASP
594 Touch® spiral plater (Don Whitley Scientific, UK). When there were not 3 flies still alive then
595 all remaining flies of a sex were used and squished in 100uL of 1x PBS per fly collected instead
596 of 300uL to keep all samples the same concentration per fly. Counts were adjusted accordingly.

597

598 **Feeding Rate Assays**

599 To measure the amount of food and bacteria consumed by males and females of different
600 genotypes, we made food containing blue dye by adding 11.2g FCF blue dye (Erioglaucine
601 disodium salt) per liter of food. We used newly eclosed flies (1-2 days post eclosion / 14 days
602 post oviposition) and separated sexes (in sterile conditions) and keep flies at a density of 10
603 flies/vial in fresh food vials. We held these flies in incubators for one day, so they would recover
604 stress of anesthesia during sexing. To introduce bacteria (or control media) into the food, we

605 pipetted 50uL of suspension or LB at an OD600 of 15 (+/- 1) into each vial and allowed the
606 suspension to absorbed into the food for 30-40 mins by keeping the vials open inside a sterile
607 hood. We next added the experimental flies and allowed them to feed for one hour. After 1 hour,
608 we anesthetized the flies on ice, then rinsed in ethanol and sterile water. Flies were homogenized
609 in 300µl of 1X PBS with a glass bead (maximum speed for 4 minutes). The homogenate was
610 centrifuged at 14000 RPM for 4 minutes and 200µl of the supernatant was used to measure
611 absorbance at 630nm. Absorbance differences were analyzed using the natural log of absorbance
612 as the response variable with genotype, sex, genotype by sex interaction, and block as
613 independent variables. Due to the blocking structure of the experiment, each treatment (no media
614 control, LB control, *P. rettgeri*) was analyzed separately.
615
616 **Desiccation**
617 Desiccation survival assays were performed on dpt^{S69} and dpt^{S69R} adult males 4-7 days post-
618 eclosion in conventionally and axenically reared flies. Ten males were placed into an empty vial
619 and closed off with a cotton flug. Each genotype had 5 vials for a total of 50 flies per line per
620 rearing condition in each replicate. The flugs were topped with silica gel (Fisher, #S684) and
621 sealed with parafilm to prevent any moisture from entering the vials. Vials were kept at 24°C on
622 12 hour day/night cycles. Survival was measured by counting flies hourly until the entire
623 population died. This was repeated once more with 50 flies per line per rearing condition in each
624 trial for a total of 100 flies with the exception for the *Δdpt* line which in total only had 30 flies
625 per condition in total across trials.
626
627 **Starvation**

628 Starvation survival assays were performed on *dpt*^{S69} and *dpt*^{S69R} adult males 4-7 days after
629 eclosion for axenically and conventionally reared flies. Ten males were placed into a vial with
630 autoclaved starvation media (1% agar). The 1% agar was used to starve flies of nutrition but not
631 desiccate them. Vials were kept at 24°C on 12-hour day/night cycles. Survival was measured by
632 counting surviving flies at three 8-hour intervals (8 am, 4 pm, and 12 am) until all flies died. This
633 was repeated twice more for a total of 3 trials with at least 40 flies per line per rearing condition
634 in each trial for a total of at least 120 flies.
635
636 **16S Sequencing**
637 Flies were reared in the lab in two distinct ways for 16S rRNA sequencing of conventionally
638 reared flies. First, flies were taken from vials of individual genotypes. This allows for any
639 moderate effects of fly genotype to equilibrate over time. Second, flies were taken from cages
640 that were started with heterozygous *diptericin* flies. These cages were started with the F1
641 progeny from crosses of *dpt*^{S69} and *dpt*^{S69R} flies and allowed to continue for 3 discrete
642 generations before flies were collected for 16S rRNA sequencing. This ensures that the
643 genotypes are exposed to the same microbes and any differences in microbiome are due to
644 genetic differences manifest in that generation.
645
646 We isolated DNA from individual flies using Gentra PureGene Tissue DNA Isolation Kit
647 (Qiagen #158388, Qiagen, Germantown, MD) following manufacturer's instructions. DNA
648 pellets were rehydrated in 10 µl DNA hydration solution. Flies from cage populations were
649 genotyped by using the dpt_cw primer pair (Table S1) followed by a restriction enzyme digest

650 using enzymes that cut DNA sequences for arginine (BccI, NEB # R0704S) or serine (AluI, NEB
651 # R0137S).
652
653 DNA concentration was measured with Qubit fluorimeter (Invitrogen). Ten flies from each
654 inbred CRISPR genome edited line (5 females and 5 males from dpt^{S69}, dpt^{S69R}, dpt^{S69R-2} (a
655 second homozygous arginine line from CRISPR/Cas9 genome editing), and *Δdpt* (1 base pair
656 deletion) and ten flies of each genotype from the cage population (5 females and 5 males of SS,
657 SR, RR genotypes) were each brought to a DNA concentration of 5 ng/µl. Libraries were
658 prepped in accordance with the 16S Metagenomic Sequencing Library Preparation protocol from
659 Illumina for the 16S V3/V4 region. Sequencing was performed on the Illumina MiSeq platform
660 using v3 300bp paired end reads. Library preparation and sequencing was performed at the
661 University of Kansas Genome Sequencing Core (Lawrence, KS, USA).
662
663 Wild flies were collected from decaying apples and pears in an apple orchard in Kansas City, KS
664 (3341 N 139th St, Kansas City, KS 66109). Flies were immediately transported back to the lab
665 and sorted by species on CO_2 and frozen at -20°C. DNA was extracted from individual flies
666 using Gentra PureGene Cell & Tissue DNA Isolation Kit (Qiagen #158388). The samples were
667 tested for species (*D. simulans* vs. *D. melanogaster*), *Wolbachia* status, and Dpt genotype using
668 primers listed in Table S1. Collections are summarized in Table S10. Libraries were prepped in
669 the same manner as the conventionally reared flies.
670
671 **16S Bioinformatic Analysis**

672 Demultiplexed reads were processed with QIIME2, v 2019.10 [83]. Primers were removed from
673 5' ends with Cutadapt using default parameters [84]. Reads were de-noised and trimmed for
674 quality with Divisive Amplicon Denoising Algorithm (DADA2) within the QIIME2
675 bioinformatics pipeline [85]. Forward and reverse reads were truncated at 280 bp and 245 bp
676 respectively. The remaining ASV table was exported from QIIME2 for further processing in R
677 [86]. Taxonomy was assigned to the ASV table using SILVA 16S rRNA gene reference
678 database, v.138 [87–89]. Reads assigned to Genus level *Wolbachia* (a *Drosophila* endosymbiont)
679 and Kingdom level Eukaryota were removed from further analysis. We also removed reads not
680 observed at least 3 times in at least 10 samples. Then, conventionally reared flies were rarefied to
681 17066 reads, while wild flies were rarefied to 10771 reads.
682
683 All statistical analysis of 16S data was performed in R (4.1.2) using the Phyloseq [90] package
684 and ggplot2 [91] package for visualization. The CRISPR and cage population data were analyzed
685 separately. For each population, alpha diversity was estimated using Shannon diversity in
686 Phyloseq using the estimate_richness() function. Bray-Curtis dissimilarity was calculated to look
687 at overall patterns of microbiome composition in Phyloseq using the ordinate() function.
688 Significance was determined at α=0.05. Fixed effects models were fit with the package lme4 [92]
689 with the fixed effects genotype + sex + genotype*sex. When necessary, P-values were adjusted
690 for multiple comparisons using FDR correction method.
691
692 **Statistical Analysis**
693 R (version 4.1.2) was used to run statistical analyses. Survival data were plotted using the R
694 package survminer [93]. The analysis was performed using the Cox proportional-hazards

695 regression model in R [94]. For longevity, significance was determined using the model:
696 Lifespan~(Genotype*Sex)/Vial+Block. For gnotobiotic bacterial load, significance was
697 determined using the model: Colonies.mL~(Genotype)*Sex+Block.
698
699
700 **Competing Interests**
701 The authors state no competing interests.
702
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710
711

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973 **Supplemental tables**

974 **Table S1. Oligonucleotide sequences used in this study.**
975

Primer name	Primer sequence	Use	Reference
wsp-81F	TGGTCCAATAAGTGATGAAGAAAC	*Wolbachia* status	Braig et al. 1998
Wsp-691R	AAAAATTAAACGCTACTCCA	*Wolbachia* status	Braig et al. 1998
Actin79B-F	CGTCTACCAGTCCATCATGA	Species identification	
Actin79B-R	AGACGGAGTACTTGCGCTC	Species identification	
Dpt_cw_F	GCCTATAAAAGAGCATCGAA	*Diptericin*	Clark and Wang, 1997
Dpt_cw_R	GCTAGACTCGGATACCAATCGAG	*Diptericin*	Clark and Wang, 1997
Dpt_gRNA18_S	CTTCGATGGCAACTCAGAACCGACG	gRNA	This work
Dpt_gRNA18_A	AAACGCTCGGTTCTGAGTTCGGATC	gRNA	This work
ssODN	CGCCACGAGATTGGACTGAATGGAGG ATATGGACAGCACTTGGGAGGACCAT ACGGCAACTCAGAACCGAGATGGAA AGTGGGAAGCACCTACACCTACAGA TTTCCGAATTTCTAAGCT	ssODN	This work

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980 **Table S2. Cox proportional hazard pairwise significance p-values for systemic infection.** P-values less than
981 0.05 are underlined.
982

P. rettgeri			
	imd-	*Δdpt*	*dptS69R*
Δdpt	4.07E-02	-	-
dptS69R	4.54E-01	0.26329	-
dptS69	1.20E-04	1.10E-09	1.30E-06
S. succinus			
	imd-	*Δdpt*	*dptS69R*
Δdpt	0.4994	-	-
dptS69R	0.0672	0.0023	-
dptS69	0.4994	0.9828	0.012
E. faecalis			
	imd-	*Δdpt*	*dptS69R*
Δdpt	0.0264	-	-
dptS69R	0.0264	1.00E-06	-
dptS69	0.2969	0.3405	2.10E-03
L. fusiformis			
	imd-	*Δdpt*	*dptS69R*
Δdpt	0.435	-	-
dptS69R	0.048	0.22	-
dptS69	0.435	0.927	0.19
L. lactis			
	imd-	*Δdpt*	*dptS69R*
Δdpt	0.7515	-	-
dptS69R	0.0474	0.0474	-
dptS69	0.1966	0.0858	0.0015
S. marcescens			
	imd-	*Δdpt*	*dptS69R*
Δdpt	<2e-16	-	-
dptS69R	<2e-16	0.1151	-
dptS69	<2e-16	0.054	0.0042

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984

985 **Table S3. Cox proportion hazard ratio pairwise p-values for systemic infections with males and females.** P-
986 values less than 0.05 are underlined.
987

P. rettgeri							
	F-*imd-*	M-*imd-*	F-*Δdpt*	M-*Δdpt*	F- dpt^{S69R}	M- dpt^{S69R}	F- dpt^{S69}
M-*imd-*	0.2560	-	-	-	-	-	-
F-*Δdpt*	0.1592	0.8278	-	-	-	-	-
M-*Δdpt*	0.2356	0.9763	0.8278	-	-	-	-
F- dpt^{S69R}	0.9763	0.2981	0.1933	0.2560	-	-	-
M-*d* dpt^{S69R}	0.0144	0.1124	0.1510	0.1124	0.0152	-	-
F- dpt^{S69}	5.60E-05	0.0014	0.0014	0.0008	8.80E-05	0.3598	-
M- dpt^{S69}	3.70E-06	9.60E-05	9.60E-05	7.70E-05	6.50E-06	0.1639	0.5982
E. faecalis							
	F-*imd-*	M-*imd-*	F-*Δdpt*	M-*Δdpt*	F- dpt^{S69R}	M- dpt^{S69R}	F- dpt^{S69}
M-*imd-*	0.0304	-	-	-	-	-	-
F-*Δdpt*	0.0036	0.8954	-	-	-	-	-
M-*Δdpt*	0.0012	0.7414	0.7414	-	-	-	-
F- dpt^{S69R}	0.7414	0.1433	0.0326	0.0158	-	-	-
M- dpt^{S69R}	0.0318	0.9686	0.8893	0.7414	0.1566	-	-
F- dpt^{S69}	0.0326	0.9686	0.8779	0.7414	0.1701	0.9686	-
M- dpt^{S69}	0.0114	0.753	0.8664	0.9686	0.0511	0.7414	0.7414
L. plantarum							
	F-*imd-*	M-*imd-*	F-*Δdpt*	M-*Δdpt*	F- dpt^{S69R}	M- dpt^{S69R}	F- dpt^{S69}
M-*imd-*	<2e-16	-	-	-	-	-	-
F-*Δdpt*	3.80E-08	<2e-16	-	-	-	-	-
M-*Δdpt*	<2e-16	8.10E-09	<2e-16	-	-	-	-
F- dpt^{S69}	<2e-16	3.20E-09	7.60E-15	0.734	-	-	-
M- dpt^{S69R}	<2e-16	1.80E-12	8.30E-15	0.372	0.197	-	-
F- dpt^{S69}	<2e-16	6.00E-15	1.00E-10	0.08	0.056	0.548	-
M- dpt^{S69}	<2e-16	5.40E-07	<2e-16	0.364	0.662	0.056	0.013

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990
991 **Table S4. P-values from Tukey HSD for axenic and gnotobiotic longevity.** P-values less than 0.05 underlined.

Axenic							
	F-*imd*-	F-*Δdpt*	F-*dpt*^{S69R}	F-*dpt*^{S69}	M-*imd*-	M-*Δdpt*	M- *dpt*^{S69R}
F-*Δdpt*	1	-	-	-	-	-	-
F-*dpt*^{S69R}	6.00E-04	<2e-16	-	-	-	-	-
F-*dpt*^{S69}	0.9447	0.8051	0.063	-	-	-	-
M-*imd*-	<2e-16	<2e-16	<2e-16	<2e-16	-	-	-
M-*Δdpt*	1.00E-04	<2e-16	1	0.0492	<2e-16	-	-
M-*dpt*^{S69R}	0.1713	0.0519	0.7467	0.8822	<2e-16	0.8133	-
M-*dpt*^{S69}	1.00E-04	<2e-16	1	0.0218	<2e-16	0.9947	0.5114
L. plantarum							
	F-*imd*-	F-*Δdpt*	F-*dpt*^{S69R}	F-*dpt*^{S69}	M-*imd*-	M-*Δdpt*	M-*dpt*^{S69R}
F-*Δdpt*	0.0064	-	-	-	-	-	-
F-*dpt*^{S69R}	<2e-16	<2e-16	-	-	-	-	-
F- *dpt*^{S69}	<2e-16	<2e-16	0.185	-	-	-	-
M-*imd*-	<2e-16	<2e-16	<2e-16	<2e-16	-	-	-
M-*Δdpt*	<2e-16	<2e-16	0.9992	0.2479	<2e-16	-	-
M-*dpt*^{S69R}	<2e-16	<2e-16	1	0.2568	<2e-16	0.9999	-
M-*dpt*^{S69}	<2e-16	<2e-16	0.9531	0.0056	<2e-16	0.5593	0.9198
P. rettgeri							
	F-*imd*-	F-*Δdpt*	F- *dpt*^{S69R}	F- *dpt*^{S69}	M-*imd*-	M-*Δdpt*	M-*dpt*^{S69R}
F-*Δdpt*	<2e-16	-	-	-	-	-	-
F-*dpt*^{S69R}	1.00E-04	0.1564	-	-	-	-	-
F-*dpt*^{S69}	<2e-16	0.5911	0.9977	-	-	-	-
M-*imd*-	<2e-16	1.12E-02	<2e-16	1.00E-04	-	-	-
M-*Δdpt*	2.34E-02	<2e-16	4.81E-01	9.95E-02	<2e-16	-	-
M-*dpt*^{S69R}	0.0013	3.63E-02	0.9997	0.9434	<2e-16	0.8429	-
M-*dpt*^{S69}	0.0938	1.00E-04	0.6415	0.2208	<2e-16	1	0.9079
L. brevis							
	F-*imd*-	F-*Δdpt*	F-*dpt*^{S69R}	F-*dpt*^{S69}	M-*imd*-	M-*Δdpt*	M-*dpt*^{S69R}
F-*Δdpt*	<2e-16	-	-	-	-	-	-
F- *dpt*^{S69R}	<2e-16	0.0348	-	-	-	-	-
F- *dpt*^{S69}	4.00E-04	9.00E-04	0.9874	-	-	-	-
M-*imd*-	<2e-16	<2e-16	<2e-16	<2e-16	-	-	-
M-*Δdpt*	1.75E-02	<2e-16	1.18E-01	6.87E-01	<2e-16	-	-
M-*dpt*^{S69R}	2.06E-02	<2e-16	0.5139	9.63E-01	<2e-16	0.9998	-
M- *dpt*^{S69}	4.02E-01	<2e-16	0.0333	2.97E-01	<2e-16	0.9803	0.9241
A. tropicalis							
	F-*imd*-	F-*Δdpt*	F-*dpt*^{S69R}	F-*dpt*^{S69}	M-*imd*-	M-*Δdpt*	M-*dpt*^{S69R}
F-*Δdpt*	<2e-16	-	-	-	-	-	-
F-*dpt*^{S69R}	0.2941	<2e-16	-	-	-	-	-
F-*dpt*^{S69}	<2e-16	0.9003	<2e-16	-	-	-	-
M-*imd*-	0.0016	<2e-16	0.6689	0.0255	-	-	-
M-*Δdpt*	0.0017	<2e-16	0.8677	4.00E-04	0.9987	-	-
M-*dpt*^{S69R}	0.9522	<2e-16	0.9472	<2e-16	0.0876	0.1404	-
M-*dptS69*	0.1403	<2e-16	1	2.00E-04	0.8812	0.9821	0.8103
L. plantarum + P. rettgeri							
	F-*imd*-	F-*Δdpt*	F-*dpt*^{S69R}	F-*dpt*^{S69}	M-*imd*-	M-*Δdpt*	M-*dpt*^{S69R}
F-*Δdpt*	0.1291	-	-	-	-	-	-
F- *dpt*^{S69R}	<2e-16	<2e-16	-	-	-	-	-
F- *dpt*^{S69}	<2e-16	<2e-16	0.3736	-	-	-	-
M-*imd*-	<2e-16	<2e-16	9.00E-04	<2e-16	-	-	-
M-*Δdpt*	<2e-16	<2e-16	0.0176	0.9872	<2e-16	-	-
M- *dpt*^{S69R}	<2e-16	<2e-16	0.998	0.807	1.00E-04	0.1564	-
M- *dpt*^{S69}	<2e-16	<2e-16	0.1446	0.9998	<2e-16	1	0.5051
L. plantarum + A. tropicalis							
	F-*imd*-	F-*Δdpt*	F-*dpt*^{S69R}	F-*dpt*^{S69}	M-*imd*-	M-*Δdpt*	M- *dpt*^{S69R}
F-*Δdpt*	0.008	-	-	-	-	-	-
F-*dpt*^{S69R}	<2e-16	<2e-16	-	-	-	-	-
F-*dpt*^{S69}	<2e-16	<2e-16	0.0632	-	-	-	-
M-*imd*-	<2e-16	<2e-16	0.9998	0.1957	-	-	-
M-*Δdpt*	<2e-16	<2e-16	0.9998	0.058	1	-	-
M-*dpt*^{S69R}	<2e-16	<2e-16	0.8864	0.7167	0.9893	0.9573	-
M-*dpt*^{S69}	<2e-16	<2e-16	0.6101	0.944	0.8792	0.7135	0.9997

992
993 **Table S5. P-values from Tukey HSD for bacteria load.** P-values less than 0.05 are underlined.

Day 2 L. plantarum							
	F-*imd-*	F-*Δdpt*	F-*dptS69R*	F-*dptS69*	M-*imd-*	M-*Δdpt*	M-*dptS69R*
F-*Δdpt*	0.8875	NA	NA	NA	NA	NA	NA
F-*dptS69R*	0.9595	0.9999	NA	NA	NA	NA	NA
F-*dptS69*	0.4774	0.7296	0.6348	NA	NA	NA	NA
M-*imd-*	9.66E-01	1.00E+00	1	0.5932	NA	NA	NA
M-*Δdpt*	0.2644	0.1289	1.53E-01	0.9983	0.1308	NA	NA
M-*dptS69R*	0.8632	1	9.99E-01	0.924	0.9982	0.4745	NA
M-*dptS69*	0.8237	0.9999	9.96E-01	0.9628	0.9928	0.5926	1
Day 10 L. plantarum							
	F-*imd-*	F-*Δdpt*	F-*dptS69R*	F-*dptS69*	M-*imd-*	M-*Δdpt*	M-*dptS69R*
F-*Δdpt*	0.8875	NA	NA	NA	NA	NA	NA
F-*dptS69R*	0.9595	0.9999	NA	NA	NA	NA	NA
F-*dptS69*	0.4774	0.7296	0.6348	NA	NA	NA	NA
M-*imd-*	9.66E-01	1.00E+00	1	0.5932	NA	NA	NA
M-*Δdpt*	0.2644	0.1289	1.53E-01	0.9983	0.1308	NA	NA
M-*dptS69R*	0.8632	1	9.99E-01	0.924	0.9982	0.4745	NA
M-*dptS69*	0.8237	0.9999	9.96E-01	0.9628	0.9928	0.5926	1
***Day 20 L. plantarum* (no imd females)**							
	F-*imd-*	F-*Δdpt*	F-*dptS69R*	F-*dptS69*	M-*imd-*	M-*Δdpt*	M-*dptS69R*
F-*Δdpt*	NA	NA	NA	NA	NA	NA	NA
F-*dptS69R*	NA	0.9978	NA	NA	NA	NA	NA
F-*dptS69*	NA	1	0.9943	NA	NA	NA	NA
M-*imd-*	NA	0.9998	1	0.9991	NA	NA	NA
M-*Δdpt*	NA	9.35E-02	0.0314	0.2254	0.0553	NA	NA
M-*dptS69R*	NA	1.00E+00	0.9807	1	0.9952	0.3207	NA
M-*dptS69*	NA	3.40E-01	0.1438	0.5173	0.2122	1	0.6305
Day 2 P. rettgeri							
	F-*imd-*	F-*Δdpt*	F-*dptS69R*	F-*dptS69*	M-*imd-*	M-*Δdpt*	M-*dptS69R*
F-*Δdpt*	0.0406	NA	NA	NA	NA	NA	NA
F-*dptS69R*	0.5572	0.9645	NA	NA	NA	NA	NA
F-*dptS69*	0.0234	0.9952	0.7777	NA	NA	NA	NA
M-*imd-*	0.5805	0.9565	1	0.7576	NA	NA	NA
M-*Δdpt*	2.10E-03	0.912	0.4482	1	0.4228	NA	NA
M-*dptS69R*	1.00E-03	0.5392	0.1887	0.9679	0.1757	0.9842	NA
M-*dptS69*	4.00E-04	0.3479	0.1066	0.8962	0.0984	0.9237	1
Day 10 P. rettgeri							
	F-*imd-*	F-*Δdpt*	F-*dptS69R*	F-*dptS69*	M-*imd-*	M-*Δdpt*	M-*dptS69R*
F-*Δdpt*	0.9999	NA	NA	NA	NA	NA	NA
F-*dptS69R*	0.1574	0.1425	NA	NA	NA	NA	NA
F-*dptS69*	0.9844	0.998	0.5859	NA	NA	NA	NA
M-*imd-*	0.0707	0.0541	0.9999	0.351	NA	NA	NA
M-*Δdpt*	0.9334	0.9767	0.57	1	0.3138	NA	NA
M-*dptS69R*	0.0086	0.0042	0.9253	0.0653	0.9903	0.0432	NA
M-*dptS69*	0.904	0.958	0.8201	0.9999	0.5938	1	0.1563
Day 20 P. rettgeri							
	F-*imd-*	F-*Δdpt*	F-*dptS69R*	F-*dptS69*	M-*imd-*	M-*Δdpt*	M-*dptS69R*
F-*Δdpt*	1	NA	NA	NA	NA	NA	NA
F-*dptS69R*	0.0241	3.00E-04	NA	NA	NA	NA	NA
F-*dptS69*	0.7316	0.4202	0.1779	NA	NA	NA	NA
M-*imd-*	0.3846	0.0835	0.5982	0.9932	NA	NA	NA
M-*Δdpt*	1	1	3.00E-04	0.4122	0.081	NA	NA
M-*dptS69R*	0.0838	0.0032	0.996	0.5558	0.9479	0.003	NA
M-*dptS69*	0.9304	0.8325	0.0445	0.9984	0.8519	0.826	0.2147

Table S5 (continued)

Day 2 L. brevis							
	F-*imd-*	F-*Δdpt*	F-*dptS69R*	F-*dptS69*	M-*imd-*	M-*Δdpt*	M-*dptS69R*
F-*Δdpt*	0.0027	NA	NA	NA	NA	NA	NA
F-*dptS69R*	2.09E-02	1	NA	NA	NA	NA	NA
F-*dptS69*	0.0855	0.9932	0.9993	NA	NA	NA	NA
M-*imd-*	3.00E-04	0.8295	0.8593	0.5336	NA	NA	NA
M-*Δdpt*	<02e-16	6.19E-01	0.7332	0.3352	1	NA	NA
M-*dptS69R*	<02e-16	0.0511	0.1029	0.026	0.8176	0.7015	NA
M-*dptS69*	1.00E-04	0.5769	0.6552	0.3136	1	0.9999	0.9518
Day 10 L. brevis							
	F-*imd-*	F-*Δdpt*	F-*dptS69R*	F-*dptS69*	M-*imd-*	M-*Δdpt*	M-*dptS69R*
F-*Δdpt*	0.9892	NA	NA	NA	NA	NA	NA
F-*dptS69R*	1	0.9836	NA	NA	NA	NA	NA
F-*dptS69*	0.6678	0.9554	0.6632	NA	NA	NA	NA
M-*imd-*	0.9941	0.6931	0.9989	0.2425	NA	NA	NA
M-*Δdpt*	8.49E-02	0.2951	0.1117	0.9918	0.0087	NA	NA
M-*dptS69R*	0.9987	1	0.9971	0.9634	0.8893	0.4734	NA
M-*dptS69*	0.0801	0.2515	0.095	0.9423	0.0119	0.9995	0.3653
Day 20 L. brevis							
	F-*imd-*	F-*Δdpt*	F-*dptS69R*	F-*dptS69*	M-*imd-*	M-*Δdpt*	M-*dptS69R*
F-*Δdpt*	0.9999	NA	NA	NA	NA	NA	NA
F-*dptS69R*	0.9998	0.9808	NA	NA	NA	NA	NA
F-*dptS69*	1	0.9997	0.9999	NA	NA	NA	NA
M-*imd-*	0.0045	2.00E-04	0.0175	0.0056	NA	NA	NA
M-*Δdpt*	0.9469	0.9854	0.7085	0.9241	0	NA	NA
M-*dptS69R*	0.9981	1	0.9528	0.9962	6.00E-04	0.9999	NA
M-*dptS69*	0.954	0.9876	0.7694	0.9363	1.00E-04	1	0.9998
Day 2 A. tropicalis							
	F-*imd-*	F-*Δdpt*	F-*dptS69R*	F-*dptS69*	M-*imd-*	M-*Δdpt*	M-*dptS69R*
F-*Δdpt*	0.9999	NA	NA	NA	NA	NA	NA
F-*dptS69R*	0.9981	0.9373	NA	NA	NA	NA	NA
F-*dptS69*	0.9993	0.9626	1	NA	NA	NA	NA
M-*imd-*	2.06E-01	0.0304	0.5635	0.5008	NA	NA	NA
M-*Δdpt*	0.5631	0.1075	0.9417	0.9084	0.9672	NA	NA
M-*dptS69R*	0.1343	0.0157	0.4295	3.72E-01	1	0.9048	NA
M-*dptS69*	0.9994	0.9634	1	1.00E+00	0.4983	0.9069	0.3698
Day 10 A. tropicalis							
	F-*imd-*	F-*Δdpt*	F-*dptS69R*	F-*dptS69*	M-*imd-*	M-*Δdpt*	M-*dptS69R*
F-*Δdpt*	0.5974	NA	NA	NA	NA	NA	NA
F-*dptS69R*	1	0.2639	NA	NA	NA	NA	NA
F-*dptS69*	0.812	1	0.5422	NA	NA	NA	NA
M-*imd-*	0.9993	0.1249	1	0.3359	NA	NA	NA
M-*Δdpt*	0.004	0.0796	2.00E-04	0.1109	1.00E-04	NA	NA
M-*dptS69R*	0.9998	0.7771	9.94E-01	0.9394	0.9524	0.0035	NA
M-*dptS69*	<2e-16	2.00E-04	<2e-16	5.00E-04	<2e-16	0.2145	<2e-16
Day 20 A. tropicalis							
	F-*imd-*	F-*Δdpt*	F-*dptS69R*	F-*dptS69*	M-*imd-*	M-*Δdpt*	M-*dptS69R*
F-*Δdpt*	0.9956	NA	NA	NA	NA	NA	NA
F-*dptS69R*	1	0.9988	NA	NA	NA	NA	NA
F-*dptS69*	1	0.998	1	NA	NA	NA	NA
M-*imd-*	0.9999	0.6771	0.9677	0.9743	NA	NA	NA
M-*Δdpt*	0.1447	3.77E-02	0.0201	1.77E-02	8.00E-04	NA	NA
M-*dptS69R*	1	9.99E-01	1	1	0.9677	0.0201	NA
M-*dptS69*	4.90E-03	1.00E-04	1.00E-04	1.00E-04	<2e-16	0.3458	1.00E-04

996 **Table S6. P-values from Tukey HSD for bacterial load from poly-association with *L. plantarum* and *P.***
997 ***rettgeri*.** P-values less than 0.05 are underlined.
998

Day 2 *L. plantarum*							
	F-*imd-*	F-*Δdpt*	F-*dptS69R*	F-*dptS69*	M-*imd-*	M-*Δdpt*	M-*dptS69R*
F-*Δdpt*	0.2226	NA	NA	NA	NA	NA	NA
F-*dptS69R*	0.0101	0.6004	NA	NA	NA	NA	NA
F-*dptS69*	<2e-16	2.00E-04	0.1471	NA	NA	NA	NA
M-*imd-*	4.21E-01	1	0.7283	0.0017	NA	NA	NA
M-*Δdpt*	0.0031	4.83E-01	1	0.0344	0.6898	NA	NA
M-*dptS69R*	<2e-16	6.00E-04	0.2442	1	0.0038	0.0725	NA
M-*dptS69*	<2e-16	4.00E-04	0.2095	1	0.0029	0.0577	1
Day 10 *L. plantarum*							
	F-*imd-*	F-*Δdpt*	F-*dptS69R*	F-*dptS69*	M-*imd-*	M-*Δdpt*	M-*dptS69R*
F-*Δdpt*	0.2226	NA	NA	NA	NA	NA	NA
F-*dptS69R*	0.0101	0.6004	NA	NA	NA	NA	NA
F-*dptS69*	<2e-16	2.00E-04	0.1471	NA	NA	NA	NA
M-*imd-*	4.21E-01	1	0.7283	0.0017	NA	NA	NA
M-*Δdpt*	0.0031	4.83E-01	1	0.0344	0.6898	NA	NA
M-*dptS69R*	<2e-16	6.00E-04	0.2442	1	0.0038	0.0725	NA
M-*dptS69*	<2e-16	4.00E-04	0.2095	1	0.0029	0.0577	1
Day 20 *L. plantarum* (no imd females)							
	F-*imd-*	F-*Δdpt*	F-*dptS69R*	F-*dptS69*	M-*imd-*	M-*Δdpt*	M-*dptS69R*
F-*Δdpt*	NA	NA	NA	NA	NA	NA	NA
F-*dptS69R*	NA	0.831	NA	NA	NA	NA	NA
F-*dptS69*	NA	0.9883	0.9977	NA	NA	NA	NA
M-*imd-*	NA	0.5334	0.9998	0.9429	NA	NA	NA
M-*Δdpt*	NA	0.9999	0.484	0.857	0.1772	NA	NA
M-*dptS69R*	NA	0.9202	1	0.9999	0.9943	0.6188	NA
M-*dptS69*	NA	0.9999	0.5746	0.8987	0.2615	1	0.7064
Day 2 *P. rettgeri*							
	F-*imd-*	F-*Δdpt*	F-*dptS69R*	F-*dptS69*	M-*imd-*	M-*Δdpt*	M-*dptS69R*
F-*Δdpt*	0.0784	NA	NA	NA	NA	NA	NA
F-*dptS69R*	0.1199	1	NA	NA	NA	NA	NA
F-*dptS69*	0.434	0.9992	0.9966	NA	NA	NA	NA
M-*imd-*	0.8069	0.912	0.8957	0.9988	NA	NA	NA
M-*Δdpt*	0.0063	0.9457	0.9972	0.8097	0.3815	NA	NA
M-*dptS69R*	0.131	1	1	0.9977	0.9101	0.9957	NA
M-*dptS69*	0.0348	0.9902	0.9996	0.9254	0.6186	1	0.9993
Day 10 *P. rettgeri*							
	F-*imd-*	F-*Δdpt*	F-*dptS69R*	F-*dptS69*	M-*imd-*	M-*Δdpt*	M-*dptS69R*
F-*Δdpt*	1	NA	NA	NA	NA	NA	NA
F-*dptS69R*	0.993	0.9967	NA	NA	NA	NA	NA
F-*dptS69*	0.9835	0.9901	1	NA	NA	NA	NA
M-*imd-*	0.998	0.9994	1	1	NA	NA	NA
M-*Δdpt*	1	0.9997	0.9485	0.9085	0.9773	NA	NA
M-*dptS69R*	1	1	0.9998	0.9992	1	0.9981	NA
M-*dptS69*	0.9911	0.9954	1	1	1	0.9393	0.9998
Day 20 *P. rettgeri* (no imd females)							
	F-*imd-*	F-*Δdpt*	F-*dptS69R*	F-*dptS69*	M-*imd-*	M-*Δdpt*	M-*dptS69R*
F-*Δdpt*	NA	NA	NA	NA	NA	NA	NA
F-*dptS69R*	NA	0.9927	NA	NA	NA	NA	NA
F-*dptS69*	NA	0.9971	0.8027	NA	NA	NA	NA
M-*imd-*	NA	0.9999	0.911	1	NA	NA	NA
M-*Δdpt*	NA	9.64E-01	1	0.5715	0.7503	NA	NA
M-*dptS69R*	NA	1	0.9896	0.9966	0.9998	0.9478	NA
M-*dptS69*	NA	0.9991	1	0.8929	0.9652	0.9998	0.9986

999

1000 **Table S7. Desiccation resistance Tukey HSD p-values.** P-values less than 0.05 are underlined. "A" refers to flies
1001 reared axenically, "C" refers to flies reared on a conventional diet.
1002

	A *dptS69*	A *Δdpt*	A *dptS69R*	A *imd-*	A OreR	A w1118	C *dptS69*	C *Δdpt*	C *dptS69R*	C *imd-*	C OreR
A *Δdpt*	0.9999	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
A *dptS69R*	0.3541	0.9978	NA	NA	NA	NA	NA	NA	NA	NA	NA
A *imd-*	0.9195	0.8332	0.003	NA	NA	NA	NA	NA	NA	NA	NA
A OreR	1	1	0.5898	0.8561	NA	NA	NA	NA	NA	NA	NA
A w1118	<2e-16	7.00E-04	<2e-16	0.007	<2e-16	NA	NA	NA	NA	NA	NA
C *dptS69*	<2e-16	0.1208	0.1473	<2e-16	1.00E-04	<2e-16	NA	NA	NA	NA	NA
C *Δdpt*	<2e-16	5.00E-04	2.00E-04	<2e-16	<2e-16	<2e-16	0.3672	NA	NA	NA	NA
C *dptS69R*	<2e-16	0.0193	0.0086	<2e-16	<2e-16	<2e-16	0.9995	0.8062	NA	NA	NA
C *imd-*	0.0057	0.7281	0.963	<2e-16	0.0242	<2e-16	0.934	0.013	0.3953	NA	NA
C OreR	0.0024	0.5452	0.8351	<2e-16	0.0105	<2e-16	0.9979	0.0653	0.8078	1	NA
C w1118	0.9996	1	0.8729	0.3974	1	<2e-16	3.00E-04	<2e-16	<2e-16	0.0781	0.0352

1003

1004 **Table S8. Starvation resistance Tukey HSD p-values.** P-values less than 0.05 are underlined. "A" refers to flies
1005 reared axenically, "C" refers to flies reared on a conventional diet.
1006

	A *dptS69*	A *Δdpt*	A *dptS69R*	A *imd-*	A OreR	A w1118	C *dptS69*	C *Δdpt*	C *dptS69R*	C *imd-*	C OreR
A *Δdpt*	0.0295	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
A *dptS69R*	<2e-16	0.0022	NA	NA	NA	NA	NA	NA	NA	NA	NA
A *imd-*	<2e-16	<2e-16	0.9288	NA	NA	NA	NA	NA	NA	NA	NA
A OreR	0.0043	1	8.00E-04	<2e-16	NA	NA	NA	NA	NA	NA	NA
A W1118	<2e-16	<2e-16	<2e-16	0.0038	<2e-16	NA	NA	NA	NA	NA	NA
C *dptS69*	5.00E-04	<2e-16	<2e-16	<2e-16	<2e-16	<2e-16	NA	NA	NA	NA	NA
C *Δdpt*	0.0055	<2e-16	<2e-16	<2e-16	<2e-16	<2e-16	1	NA	NA	NA	NA
C *dptS69R*	<2e-16	0.0614	0.998	0.3139	0.0407	<2e-16	<2e-16	<2e-16	NA	NA	NA
C *imd-*	2.00E-04	0.9988	0.0306	<2e-16	0.9995	<2e-16	<2e-16	<2e-16	0.3851	NA	NA
C OreR	0.7443	0.9473	<2e-16	<2e-16	0.828	<2e-16	<2e-16	<2e-16	1.00E-04	0.325	NA
C w1118	<2e-16	<2e-16	1.00E-04	0.0374	0	1	<2e-16	<2e-16	<2e-16	<2e-16	<2e-16

1007
1008
1009

1010
1011 **Table S9. Conventional longevity Cox proportional hazards regression p-values.** P-values less than 0.05 are
1012 underlined. "F" and "M" refer to males and females, respectively; "het" refers to heterozygotes for serine/arginine.
1013

	F het	F *imd-*	F *Δdpt*	F *dptS69R*	F *dptS69*	M het	M *imd-*	M *Δdpt*	M *dptS69R*
F *imd-*	1	NA	NA	NA	NA	NA	NA	NA	NA
F *Δdpt*	1	1	NA	NA	NA	NA	NA	NA	NA
F *dptS69R*	<2e-16	<2e-16	<2e-16	NA	NA	NA	NA	NA	NA
F *dptS69*	<2e-16	3.00E-04	<2e-16	0.6785	NA	NA	NA	NA	NA
M het	<2e-16	<2e-16	<2e-16	0.9797	0.0443	NA	NA	NA	NA
M *imd-*	0.1346	0.3182	0.0498	0.0025	0.5097	0	NA	NA	NA
M *Δdpt*	<2e-16	<2e-16	<2e-16	0.948	0.9977	0.1163	0.0321	NA	NA
M *dptS69R*	<2e-16	<2e-16	<2e-16	0.0622	<2e-16	0.4311	<2e-16	<2e-16	NA
M *dptS69*	<2e-16	<2e-16	<2e-16	0.9984	0.9882	0.5841	0.0492	1	0.0043

1014

Table S10. Genotyped wild caught *D. melanogaster.*

Wolbachia/Sex	SS	SR	RR
Wolbachia+ female	203	64	8
Wolbachia+ male	369	77	9
Wolbachia- female	68	21	2
Wolbachia- male	102	31	1
Total	742	193	20

Supplemental Figures

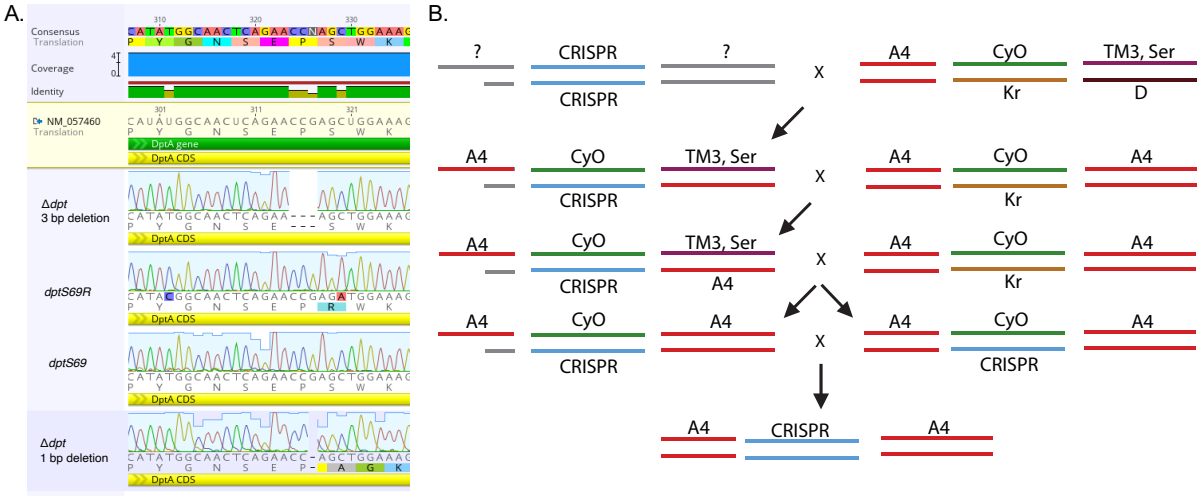

Figure S1. CRISPR/Cas9 genome editing of the *dpt* locus

A) Alignment of CRISPR/Cas9 genome edited lines. CRISPR/Cas9 genome edited lines aligned to consensus sequence. Genotype/line name is indicated on the left-hand side of the figure. B) Crossing schematic for CRISPR clean up. A modified Bloomington stock # 7198 was used for CRISPR injections. The line contained *CyO*/*Kruppel* balanced on the 2nd chromosome, and *serrate*/*Dichaete* balanced on the 3rd chromosome. Our version, 7198^{A4}, was provided by Stuart Macdonald and has the DSPR (King et al. 2012) A4 line's X chromosome instead of the *w*[*] from the original 7198.

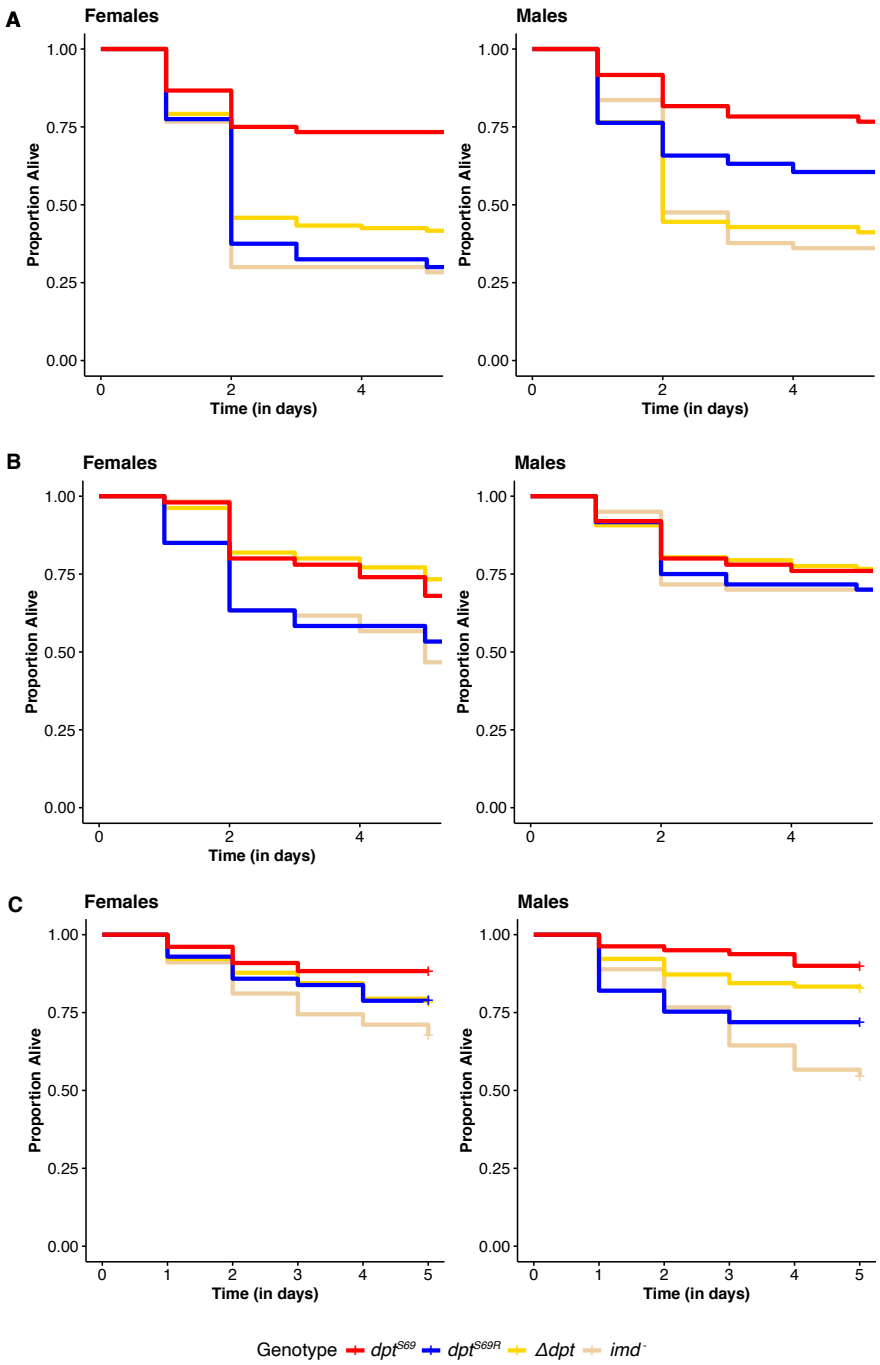

Figure S2. Male vs. female survival after systemic infection

Systemic infections were repeated with females and males. A) *P. rettgeri* and B) *E. faecalis* are normal systemic infection bacteria in *Drosophila*. C) *L. plantarum* was used to test differences in systemic based on sex based on the results from gnotobiotic longevity.

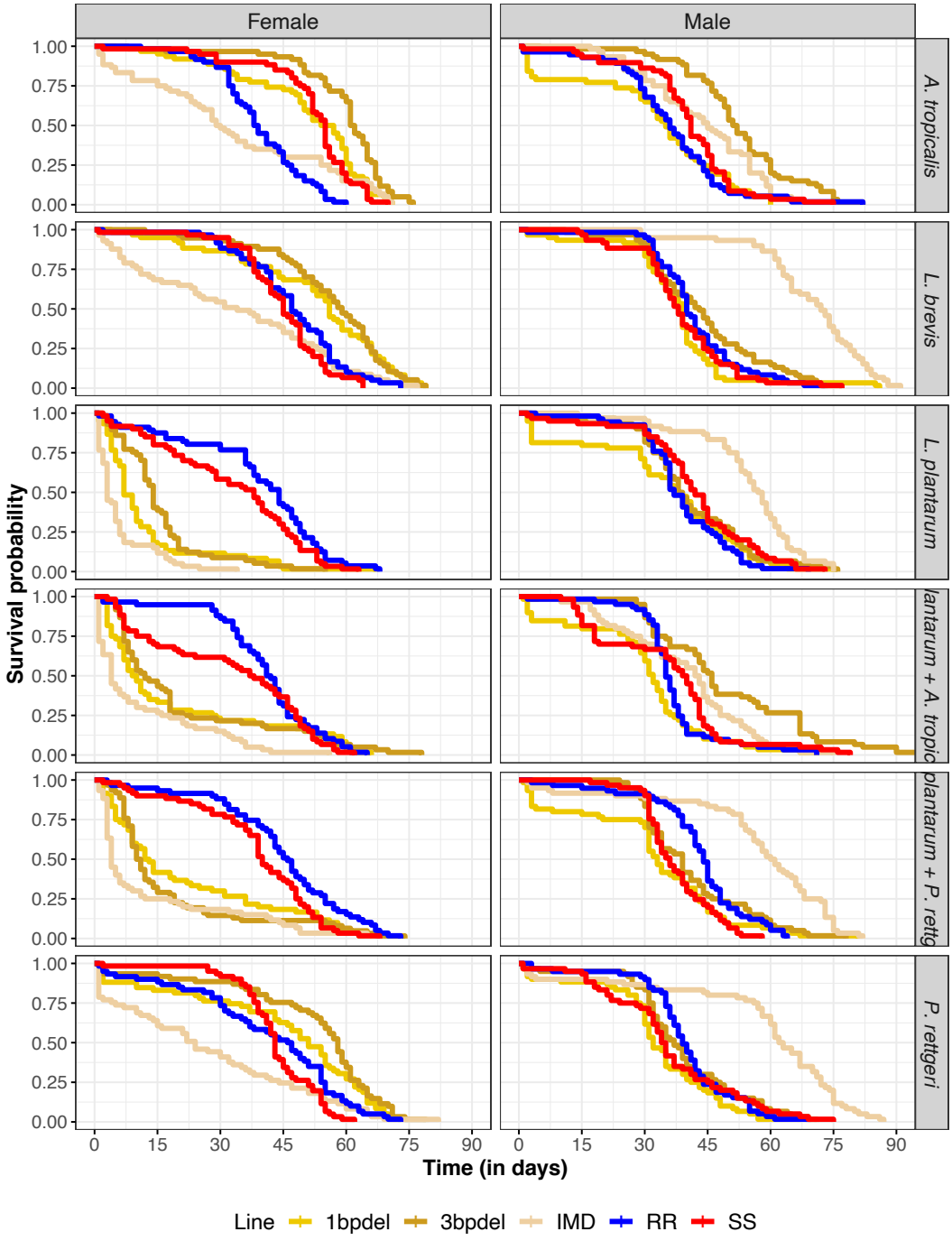

Figure S3. Gnotobiotic longevity
Survival plots of all bacteria. Dpt null lines have been separated into the two lines used, 1 base pair deletion and 3 bp deletion.

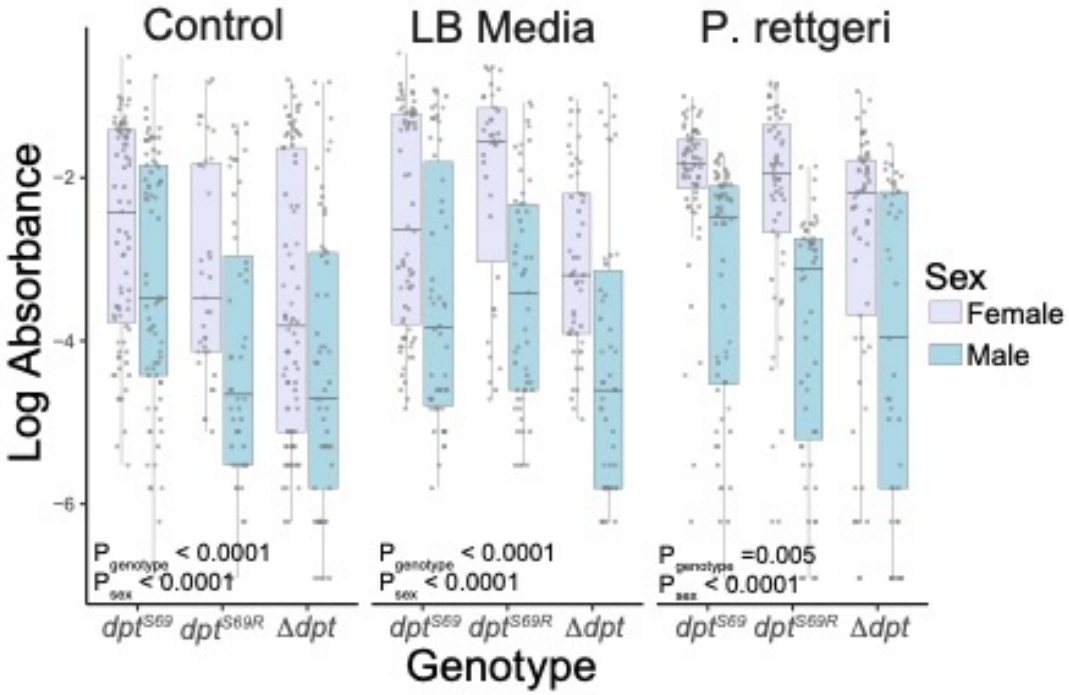

Figure S4. Feeding Rates
Female flies eat more food (as measured by blue dye ingestion) than males, but genotype also influences feeding. Each genotype has more than 100 individuals per treatment per sex. Control refers to no added media, LB Media is the addition of sterile MB, and *P. rettgeri* is the addition of *P. rettgeri* at a concentration of OD_{600} = 15 (±1). Absorbance was measured at 630nm.

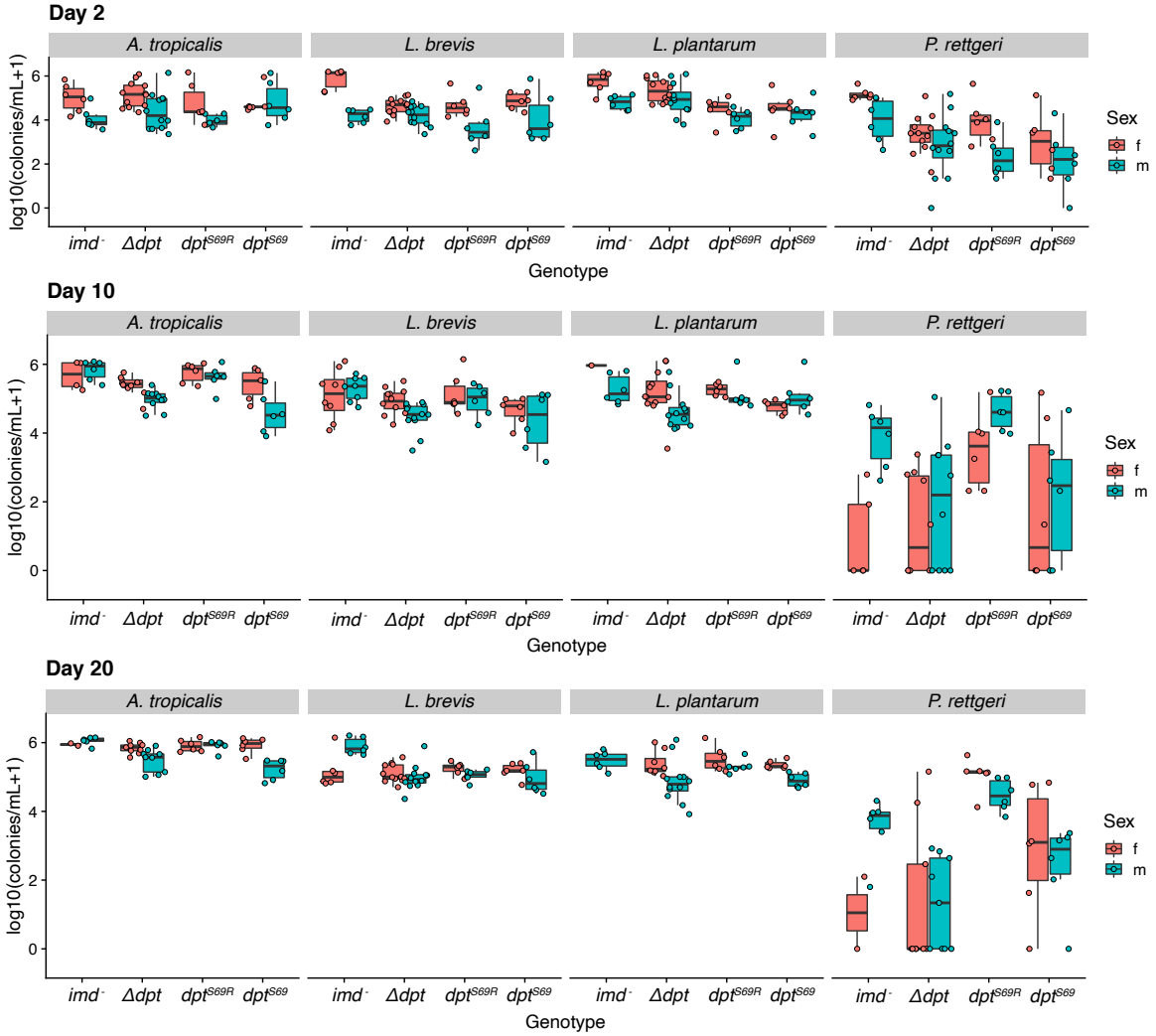

Figure S5. Bacterial load at day 2-, 10-, and 20-days post feeding
All bacteria were plated on MRS media except for *P. rettgeri* that was plated on LB media to grow colonies. Significance can be found in Table 2.S6. Individual points represent a pool of 3 flies. There is no *imd*- female bar for *L. plantarum* on day 20 because all female flies had died at this timepoint.

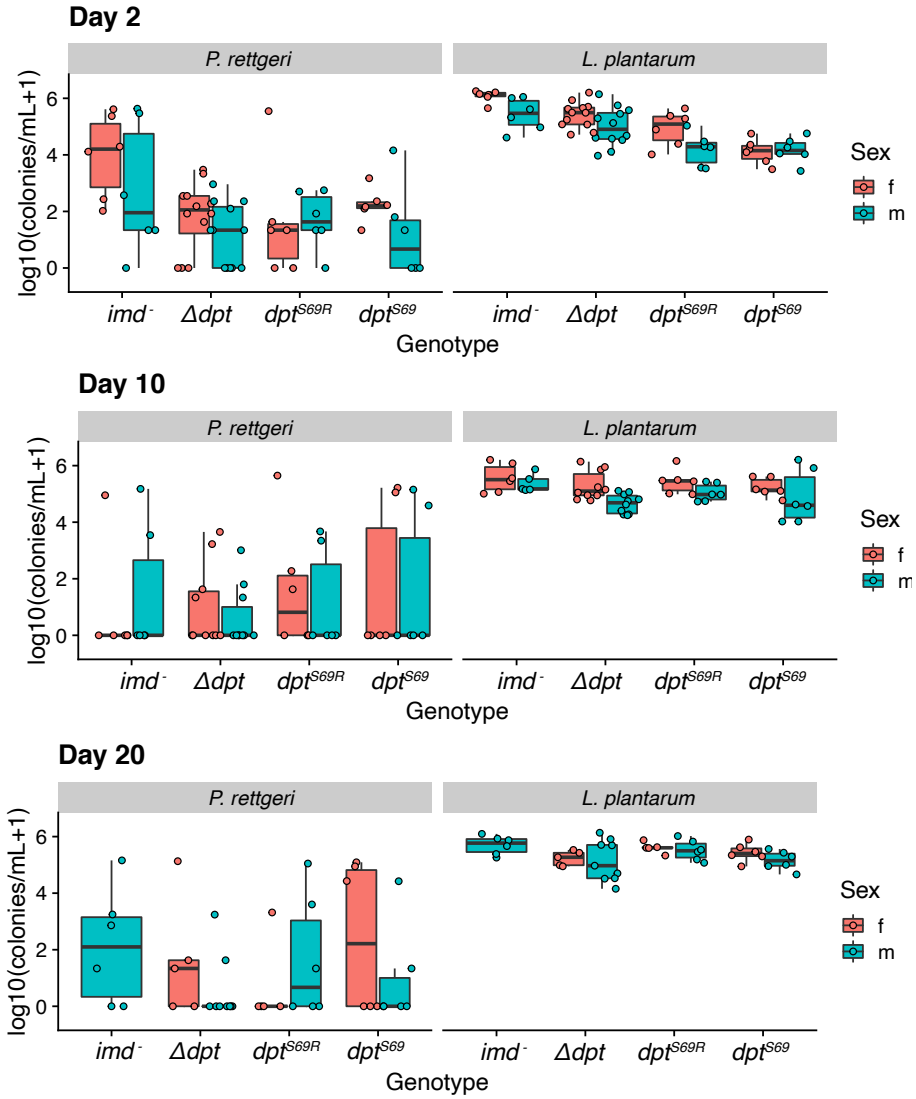

Figure S6. Bacterial load of *L. plantarum* and *P. rettgeri* poly-association
The flies were co-feed with *L. plantarum* and *P. rettgeri* and plated on MRS and LB respectively to select for the different bacteria. There is no *imd*- female bar on day 20 because all flies had died at this timepoint.

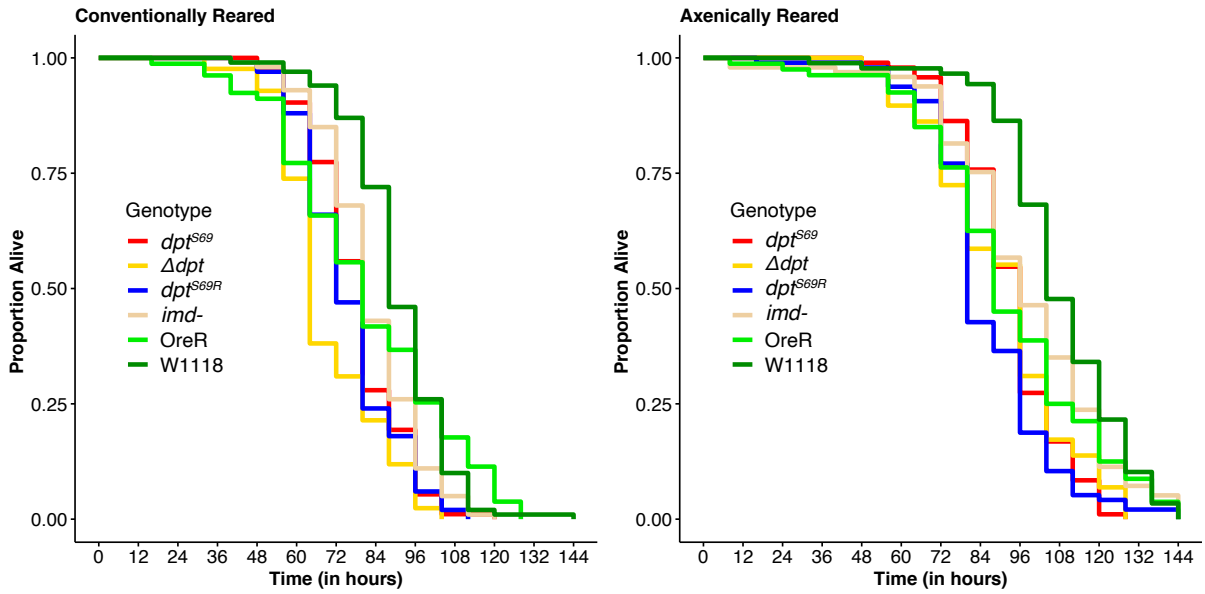

Figure S7. Desiccation stress survival with additional wildtype lines (OreR and W1118)
OreR and W1118 are standard *Drosophila melanogaster* lab stocks. They are both homozygous serine but have different resistance to desiccation stress than dpt^{S69}.

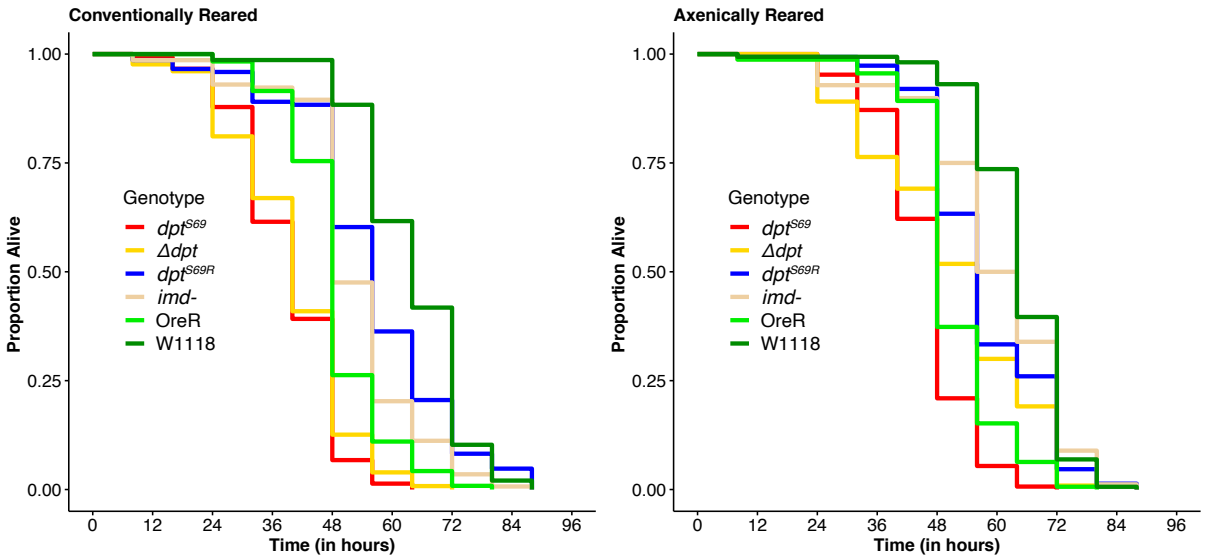

Figure S8. Starvation with additional wildtype lines (OreR and w1118)
OreR and W1118 are standard *Drosophila melanogaster* lab stocks. They are both homozygous serine but have different resistance to starvation stress than dpt^{S69}.

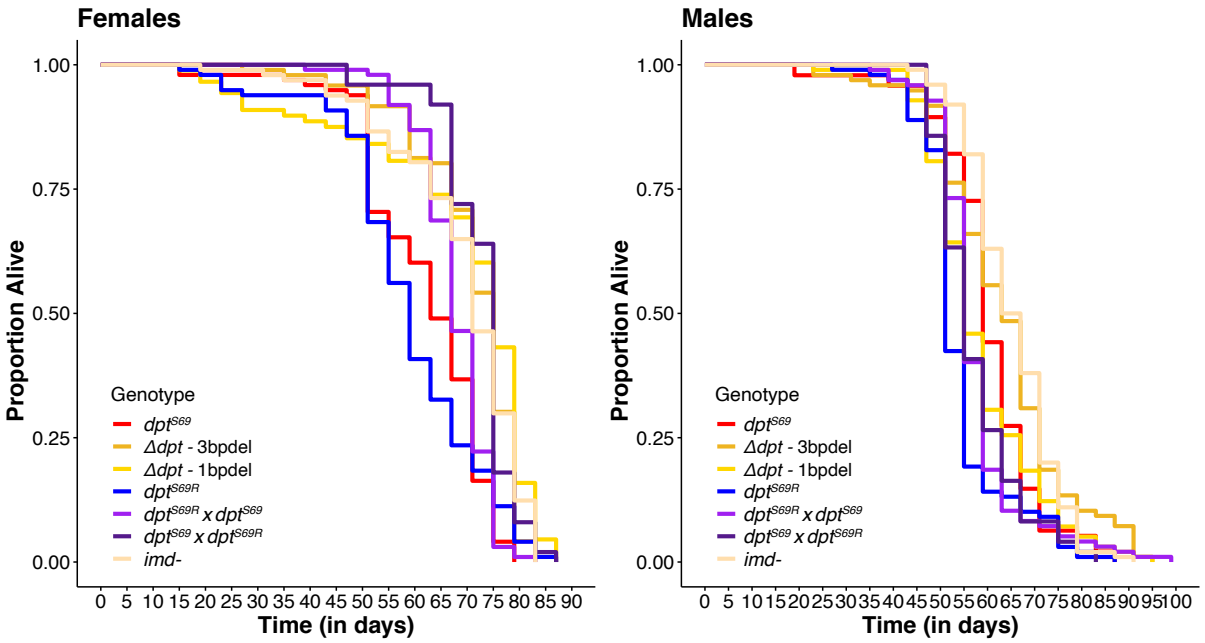

Figure S9. Longevity with separated *Δdpt* lines and heterozygotes

Two *Δdpt* lines were used. One with a 1 base pair deletion and the other with a 3 base pair deletion. These lines were combined for ease of viewing in Fig 4C. Heterozygotes were made with reciprocal crosses